Study of the Regulation of Goldfish *Carassius auratus* Prolactin Gene Expression

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Abstract

Prolactin (PRL) is specifically expressed by lactotrophs of the anterior pituitary in virtually all vertebrates, performing functions as diverse as osmoregulation and stimulation of mammalian lactation. In teleosts, the osmoregulatory actions of PRL have been well studied in euryhaline fish as an important endocrine secretion in fresh water adaptation. However, in fresh water fish, the role of PRL is less clear. It has been suggested that PRL is more involved in reproduction in such species.

The mechanism with which PRL expression is restricted to the lactotrophs of the anterior pituitary has also been investigated. It is of interest to determine whether common regulatory mechanisms of PRL gene expression exist between teleosts and mammals, and between euryhaline teleosts and freshwater teleosts. To this end, we have investigated the goldfish PRL (gfPRL) gene and its promoter. Two cDNAs of PRL, P1A and P8A, have been identified previously. Primers designed from both the P1A and P8A sequences were used to amplify PRL gene and obtained a gene fragment corresponding to P8A with size around 3.2kb. The gene fragment is composed of 5 exons and 4 introns. This genomic organization is similar to other mammalian and teleostean species. Besides, the gene was found to contain 600 bp 5-'flanking sequence, of which several possible Pit-1 binding sites were identified. The activity of the promoter of the gfPRL gene fused to the luciferase reporter gene was studied using Pit-1 expressing rat pituitary GH3 and GH4ZR7 cells and other cell types. Deletion analysis of the gfPRL promoter showed that there is a repression region, of which the repressive effect was elminated by the most upstream sequence. Besides, *in vitro* study showed that the gfPRL promoter exhibited negative response to both thyrotropin-releasing hormone (TRH) and dopamine (DA).

We also investigated the correlation of the reproductive cycle and the serum PRL and growth hormone (GH) level as well as the receptor mRNA expression level in the

following goldfish tissues: brain, liver, kidney, testis and ovary. The results showed that goldfish became sexually matured in Feburary (late winter) while serum PRL and GH rise to peak in July and August (summer). The PRL receptor and GH receptor mRNA expression level of the brain, kidney and liver showed no clear relationship with respect to the sexual maturity cycle. However, it is obvious that the receptor mRNA level of the ovary and testis rise to peak level in January and February, coinciding with the sexual maturity of goldfish. It is thus suggested that PRL and GH play an important role in fish reproduction such as gonad development.

摘要

催乳素是由脊椎動物垂叶催乳素細胞 表達的, 其功能十分多樣化, 包括滲透壓調節, 及刺激哺乳動物泌乳等。在鹽性硬骨魚中, 催乳素對滲透壓的調節已被擴泛研究。它的主要功用爲幫助廣鹽性硬骨魚適應淡水環境。然而對於淡水硬骨魚來說, 催乳素的作用還不十分清楚, 推測它可能對生殖有作用。

催乳素的細胞特異性表達機制在哺乳類動物也同樣被擴泛研究。在硬骨魚類和哺乳類, 廣鹽性魚和淡水魚之間, 會否出現相同的調控機制? 爲此, 我們研究了金魚催乳素的基因及其啓動子。我們克隆了金魚催乳素的兩種反向轉錄脫氧核糖核酸, P1A 和 P8A。根據 P1A 和 P8A 序列所設計的引物抄增了對應於 P8A 的基因片段,長達 3.2kb。該基因包含有 5 個外顯子和 4 個內顯子。這基因結構與其它哺乳動物和魚類的相同。該基因還含有一段 600bp 的 5'側翼序列, 其中含有幾個 Pit-1 結合位點。 通過將該啓動子與熒光素酶報告基因融合, 我們研究了它在表達 Pit-1 的大老鼠垂體 GH3 和 GH4ZR7 細胞以及其它細胞糸中的活性。缺失分析研究顯示, 金魚催乳素啓動子中有一被上游序列所掩蓋的抑制區。另外, 體外研究顯示, 甲狀腺釋放素(TRH)和多巴胺(DA)對該啓動子有抑制作用。

我們也研究了金魚不同組織中, 括包腦, 肝, 胃, 雄性和雌性性腺中, 催乳素, 生長激素及其受體 mRNA 表達水平與其生殖周期的關係。結果顯示, 金魚於二月進入性成熟期,而催乳素和生長激素在 7,8 月達到表達高峰, 其受體 mRNA 在腦, 肝, 胃中的表達與其性成熟周期無明顯關係。然而, 受體 mRNA 在雄性和雌

性性腺中的表達於 1,2 月達到高峰, 與金魚的性成熟期一致。表明催乳素和生長激素在金魚的生殖, 如性腺發育, 中起著重要作用。

Abbreviations

aa	amino acid(s)
AC	adenylyl cyclase
Amp	ampicillin
APS	ammonium persulfate
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate
Bis-arcylamide	N',N'-methylene bisacrylamide
bp	base pair(s)
BSA	bovine serum albumin
BTE	basal transcription element
BTF	basal transcription factor
C	chloramphenicol
cDNA	complementary DNA
Ci	Curie
CIAP	calf intestine alkaline phosphatase
cpm	counts per minute
CREB	cAMP-responsive element binding protein
DA	dopamine
DEPC	diethyl pyrocarbonate
ddH_2O	double distilled water
dNTP	deoxyribonucleotide 5'-triphosphate
DNA	deoxyribonucleotide
DNAase	deoxyribonuclease
E. coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
ERE	estrogen responsive element
ERK	extracellular regulated kinase
EtBr	ethidium bromide
FPLC	Fast Performance Liquid Chromotography
FCS	fetal calf serum
g	gravity

gf	goldfish
GH	growth hormone
GHR	growth hormone receptor
GSH	reduced form of glutathione
GSI	gonadosomatic index
GSSG	oxidized form of glutathione
h	hour(s)
IGF-I/II	Insulin-like growth factor I/II
IgG	Immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
JAK	Janus tyrosine kinases
kb	kilobase pair(s)
kDA	kilodalton
L	litre(s)
LSF-1	lactotroph-specific factor
mA	milliampere
MAPK	mitogen activated protein kinase
min	minute(s)
n	native
NBT	nitro blue tetrazolium
O.D.	optical density
ori	origin of replication
p	plasmid designation
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIF	prolactin-inhibitory factor(s)
PKC	protein kinase C
PL	placental lactogen
PLC-β	phospholipase C β
PMSF	phenylmethyl sulfonyl fluoride
PRF	prolactin-releasing factor(s)
PRL	prolactin
PRLR	prolactin receptor

PTX	pertussis toxin
PVDF	polyvinylidene difluoride
r	recombinant
RPD	rostral pars distalis
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second(s)
SEM	Standard Error of Mean
SL	somatolactin
SS	somatostatin
STAT	Signal Transducer and Activator of Transcription proteins
TAE	Tris-acetate-EDTA-buffer
TBS	Tris-buffered saline
TCA	Trichoroacetic acid
TEMED	N,N, N',N'-tetramethyl ethyenediamine
TRH	Thyrotopin-releasing hormone
Tris	Tris (hydroxymethyl) aminomethane
UTR	untranslated region
UV	ultraviolet
V	volt(s)
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Abbreviation Table for Amino Acids

Amino Acid	Three-Letter	One-Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phemylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter One

General Introduction

1.1 Structures of PRL

All vertebrates produce prolactin (PRL) and their structures have been revealed by the cDNAs cloned from several species (Cooke *et al.*, 1980; Cooke *et al.*, 1981; Mercier *et al.*, 1989; Miller *et al.*, 1981; Nicoll *et al.*, 1986; Rentier-Delrue *et al.*, 1989; Sinha, 1995). With the exception of fish, all PRLs identified so far have 197-199 amino acids (aa) and contain six cysteines forming three intramolecular disulfide bonds. Fish PRLs, however, are shorter than mammalian PRLs. They lack a dozen residues at the N-terminus and thus do not have the first disulfide bridge (Rentier-Delrue *et al.*, 1989). The physiological significance of this deletion is not clear, but the deleted fragment may be responsible for the actions of PRL unique to mammals such as stimulation of some aspect of mammary function.

In tiliapia, two distinct PRLs have been isolated, PRL-I (tPRL188) and PRL-II (tPRL177). They differ by their length (11aa), composition (69% aa identity), and biological activities (Rentier-Delrue *et al.*, 1989). In contrast, two goldfish PRL cDNAs, P1A and P8A, were identified encoding for the same mature hormone with 187aa. The gfPRL aa sequence shares sequence identities of 95% with other carp PRLs, 73-74% with salmon PRLs, and only 65% and 57% with tilapia PRL-I (tPRL188) and PRL-II (tPRL177) respectively (Chan *et al.*, 1996).

A comparison of the aa sequences of PRL from different species shows varying degrees of sequence homology (Sinha, 1995), reflecting to a great extent the order of the phylogenetic relationships. Some 32 residues are conserved among the different species (Watahiki *et al.*, 1989). These conserved residues cluster in four distinct regions of the

molecule that stretch between position 18-32, 58-72, 83-98 and 160-199 (Watahiki *et al.*, 1989). These domains are considered to form determinants for specific binding to receptors and may be critical for PRL-specific activities.

The three-dimensional (3D) structure of the PRL molecule has not been experimentally determined, but since there is substantial sequence homology between PRL and GH, Goffin *et al.* (1995) has developed a 3D model of human PRL based upon the 3D structure of porcine GH (Abdel-Meguid *et al.*, 1987; de Vos *et al.*, 1992). Under this model, PRL is composed of four α-helices organized in an antiparallel four-helix bundle, with the receptor-binding sites located on the same side of the folded protein (Fig.1.1).

Mammalian PRL is a single chain polypeptide of 23 kDa with three intramolecular disulfide bridges, N-linked (in some species, O-linked) glycosylation sites, and three phosphorylation sites. Many variants of PRL are known and could theoretically be formed by transcriptional or translational mechanisms (Fig.1.2). Since the PRL gene has several potential splice sites, variants could arise by differential splicing. An alternatively spliced PRL mRNA, presumably missing exon 4 and encoding a PRL molecule of 137 aa, has been reported in the brain (Emanuele *et al.*, 1992). Retention of an intron, potentially resulting in a 28 kDa molecule, has also been reported (Sinha, 1995).

However, translation products of these mRNAs have not yet been identified. Instead, most if not all PRL variants are formed by posttranslational modifications and differ in sizes and functional groups. Larger forms of PRL are mostly formed by aggregation. In general, only some forms retain PRL-like activities while others have unique properties or no known

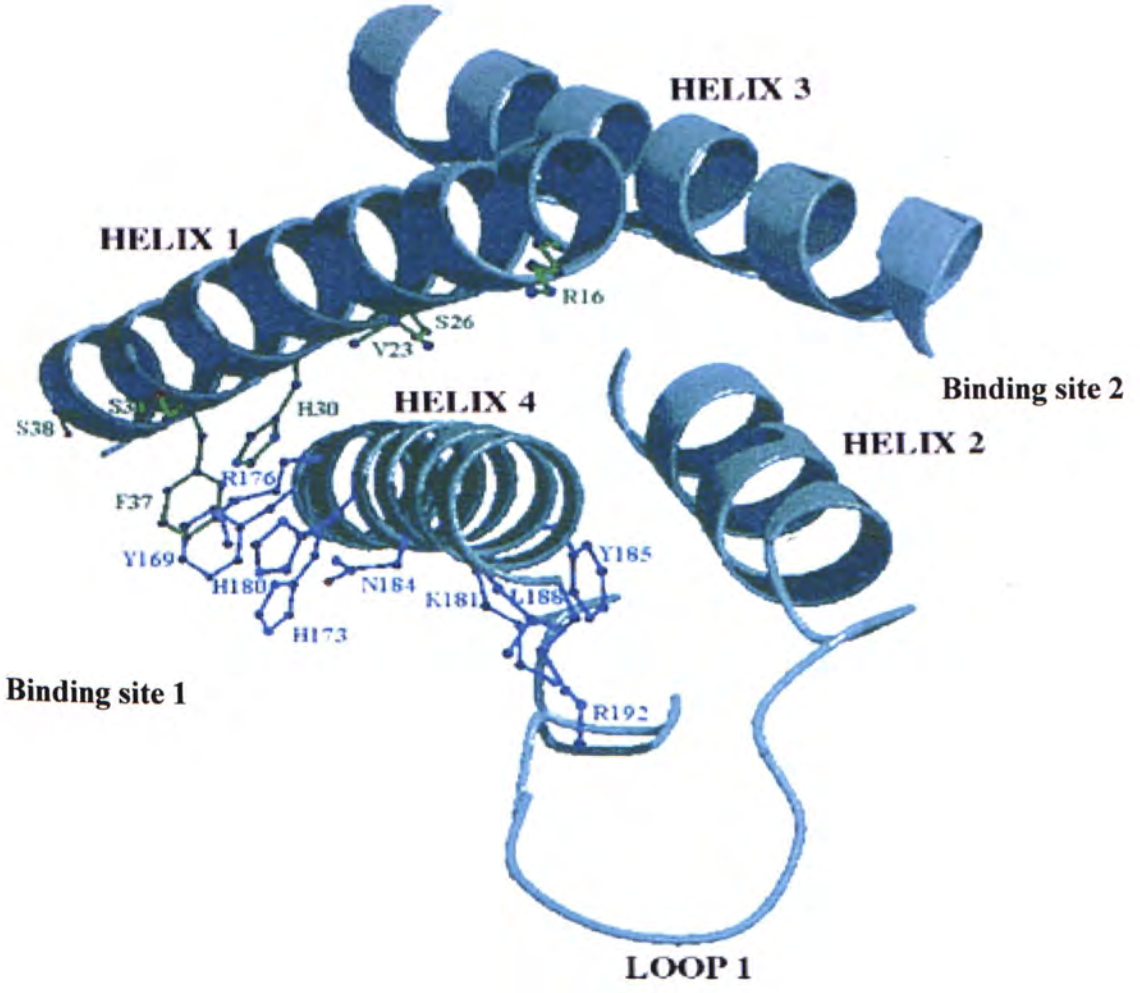


Fig.1.1. The theoretical 3D structure of human PRL. The structure has been modeled using the crystallographic coordinates of porcine GH (Abdel-Meguid *et al.*, 1987). The four-helix bundle scarfold is assumed to be shared by all members of the PRL/GH/placental lactogen (PL) family. Binding site 1 is composed of helix 1, loop 1, and helix 4. Binding site 2 is located around the cleft defined by helices 1 and 3 (Goffin *et al.*, 1995).

functions. Smaller molecular mass variants of PRL are produced by proteolytic cleavage, both at the sites of synthesis (Casabiell *et al.*, 1989; Powers and Hatala, 1990) and at some target tissues (Baldocchi *et al.*, 1992; DeVito *et al.*, 1992). Two variants, a 22 kDa and a 16 kDa isoform, are of particular interest. The 22 kDa form, PRL1-173, is generated by Kallikerin, a trypsin-like serine protease that is abundant in lactotrophs (Powers and Hatala, 1990). It cleaves PRL at Arg174-Arg175, followed by the removal of the last Arg residue by carboxypeptidase. The production of this variant is sex-dependent and is stimulated by estrogen and suppressed by dopamine (DA) (Anthony *et al.*, 1993). The higher release levels in females than in males suggest a potential role of this PRL1-173 in female reproduction.

The 16 kDa PRL is of interest because of its antiangiogenic activity. Its formation proceeds in two steps: cleavage by a cathepsin D-like protease around residues 145-149, which generates a two-chain molecule joined by a disulfide bond, followed by a reduction yielding of 16 kDa (PRL1-143) and 8 kDa fragments. The 16 kDa PRL was detected in the hypothalamus (Torner *et al.*, 1995), pituitary, and serum (Sinha *et al.*, 1985), accounting for about 1% of total secreted PRL. It may have a higher lactogenic and mitogenic potency in the mammary gland than intact PRL (Mittra, 1980), but this hypothesis has no prove evidence at all. In fact, the 16 kDa PRL binds only weakly to PRL receptors (Clapp *et al.*, 1989) and because it lacks the putative fourth α-helix, it may have a very different configuration. The 16 kDa PRL inhibits basal and fibroblast growth factor (FGF)-stimulated growth of capillary endothelial cells (Clapp *et al.*, 1993). This antiangiogenic activity, shown both *in vivo* and *in vitro,* is mediated by a high-affinity receptor distinct from the PRL receptor (Clapp and Weiner, 1992).

Glycosylated PRL is detected in pituitary and plasma, composing 1%, 15% and 50% of total PRL in bovine, human, and porcine pituitaries, respectively (Sinha *et al.*, 1985). There

is evidence for constitutive, rather than regulated, secretion of glycosylated PRL (Pellegrini *et al.*, 1990). The carbohydrate moieties vary among species, and their heterogeneity accounts for differences in bioactivity, immunoreactivity, receptor binding, and metabolic clearance rate (MCR) (Sinha *et al.*, 1985). Glycosylation often decreases PRL bioactivity, although unique physiological functions of glycosylated PRL have not been identified. PRL can also be mono-or diphosphorylated on serine and/or theonine residues, a modification that results in charge variability (Ho *et al.*, 1993). The ratio of phosphorylated/nonphosylated forms is altered during the estrous cycle and pregnancy (Ho *et al.*, 1993). Since phosphorylated PRL inhibits the release of native PRL from GH3 cells (Krown *et al.*, 1992), it may have an autocrine/paracrine function.

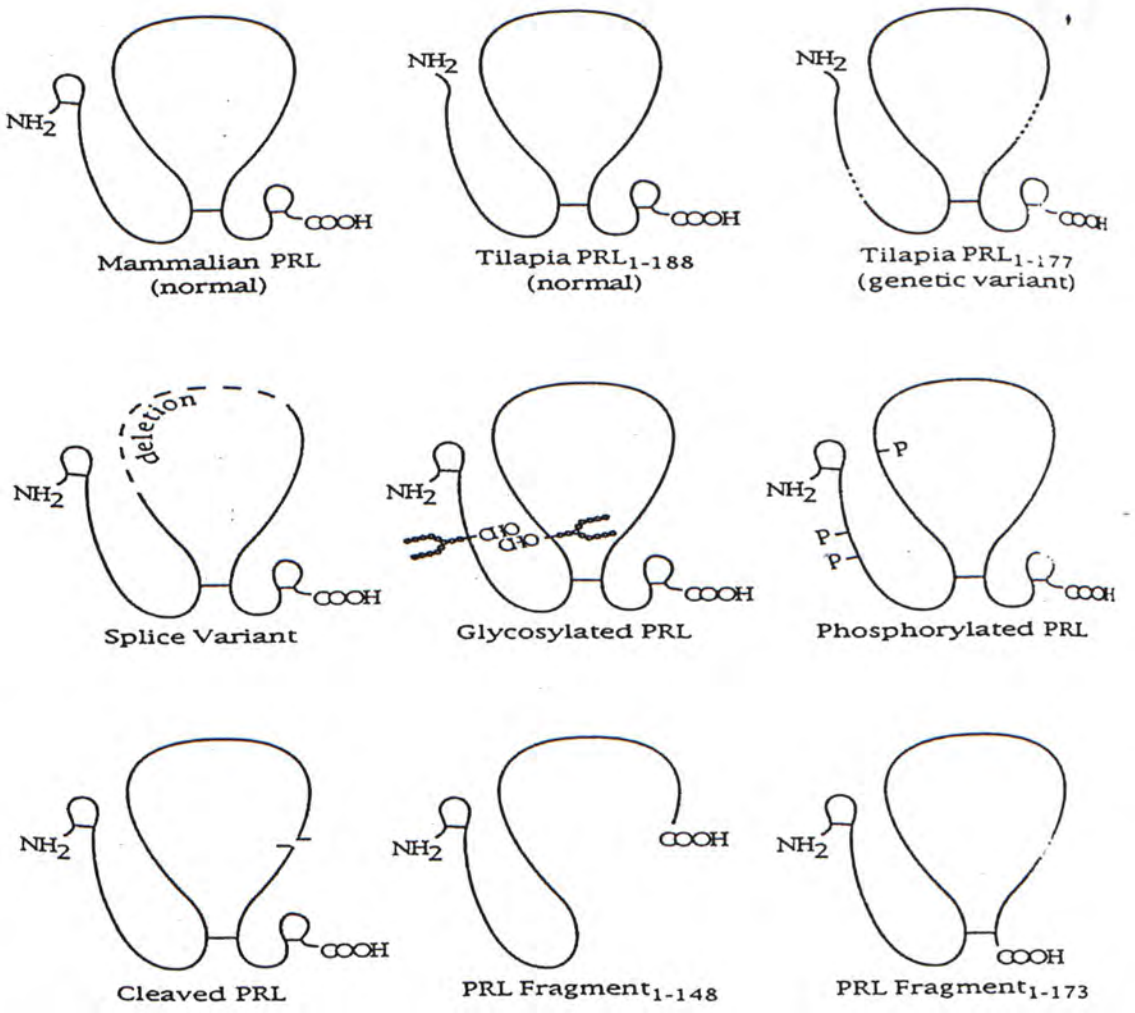


Fig.1.2. Schematic diagram of PRL molecule and some of its structural variants. The two tilapia PRLs shown represent the two genetic variants of PRL in this species; in mammals, genetic variants have not been identified. The protein representing the splice variant has not yet been identified (only have evidence from mRNA). CHO refers to the N-linked carbohydrate moieties attached to asparagine at position 31 in human, monkey, ovine, porcine, and dromedary PRLs, and at position 60 in crocodile and alligator PRLs; P represents the site of phosphorylation of serine residues at positions 26, 31 and 90. Broken lines indicate the deletion of aa residues from the genetic variant of tilapia PRL, and from the putative splice variant. The nick in the large disulfide loop shows one of the proteolytic cleavage sites in PRL; reduction of the disulfide bond of such a cleaved form gives rise to fragment1-148 (16 kDa) (Sinha, 1995).

1.2 PRL receptor and its mechanism of action

The PRL receptor, originally cloned from rat liver, encodes a 291 aa protein that is considerably shorter than the homologous GH receptor. It was later discovered that the rat has a second, 'long' receptor form that was cloned from the ovary and contains 591 aa (Kelly *et al.*, 1991). A third, 'intermediate' form, containing 391 aa, has been detected in the Nb2 transformed rat T cell line (Ali *et al.*, 1991). It is a truncated form of the long receptor missing the last 198 aa due to a deletion in the last exon. In addition to the membrane-bound receptors, soluble PRL binding proteins were also described in mammary epithelial cells (Berthon *et al.*, 1987) and milk (Postel-Vinay *et al.*, 1991a).

The PRL receptor belongs to the hematopoietic receptor family that includes receptors specific for GH, cytokines, and growth factors (Nicoll *et al.*, 1986; Kelly *et al.*, 1991). They all contain single hydrophobic trans-membrane domain that divides the receptor into an extracellular ligand binding domain with an intracellular domain (Kelly *et al.*, 1991)(Fig.1.3). Features common to the extracellular domain include four paired cysteine residues and a WSXWS (tryptophan-serine-any aa-tryptophan-serine) motif, both required to form the ligand-binding pocket. The cytoplasmic domains of these receptors differ in size and structure. A hydrophobic proline-rich motif (Box 1), located near the transmembrane region, is essential for signal transduction for all ligands studied (Dinerstein *et al.*, 1995; Horseman and Yu-Lee, 1994).

The PRL receptor and several other hematopoietic receptors also contain a less conserved cytoplasmic region, denoted Box 2 (Leung *et al.*, 1987; Lebrun *et al.*, 1995). The long and short PRL receptor isoforms in the rat have identical extracellular domains but differ in the length and sequence of the intracellular domain. These differences appear to be the result of alternative splicing of a single PRL receptor gene. Both isoforms of the PRL

receptor exist in most rat tissues, and their ratio is altered under some conditions (Di Carlo *et al.*, 1995; Nagano and Kelly, 1994). When transfected into COS cells, the short PRL receptor does not stimulate milk protein gene promoter (Lebrun *et al.*, 1995). Although the exact function served by each isoform is unknown, one might predict that they are coupled to different signal transduction pathways and account for some of the pleiotropic actions of PRL.

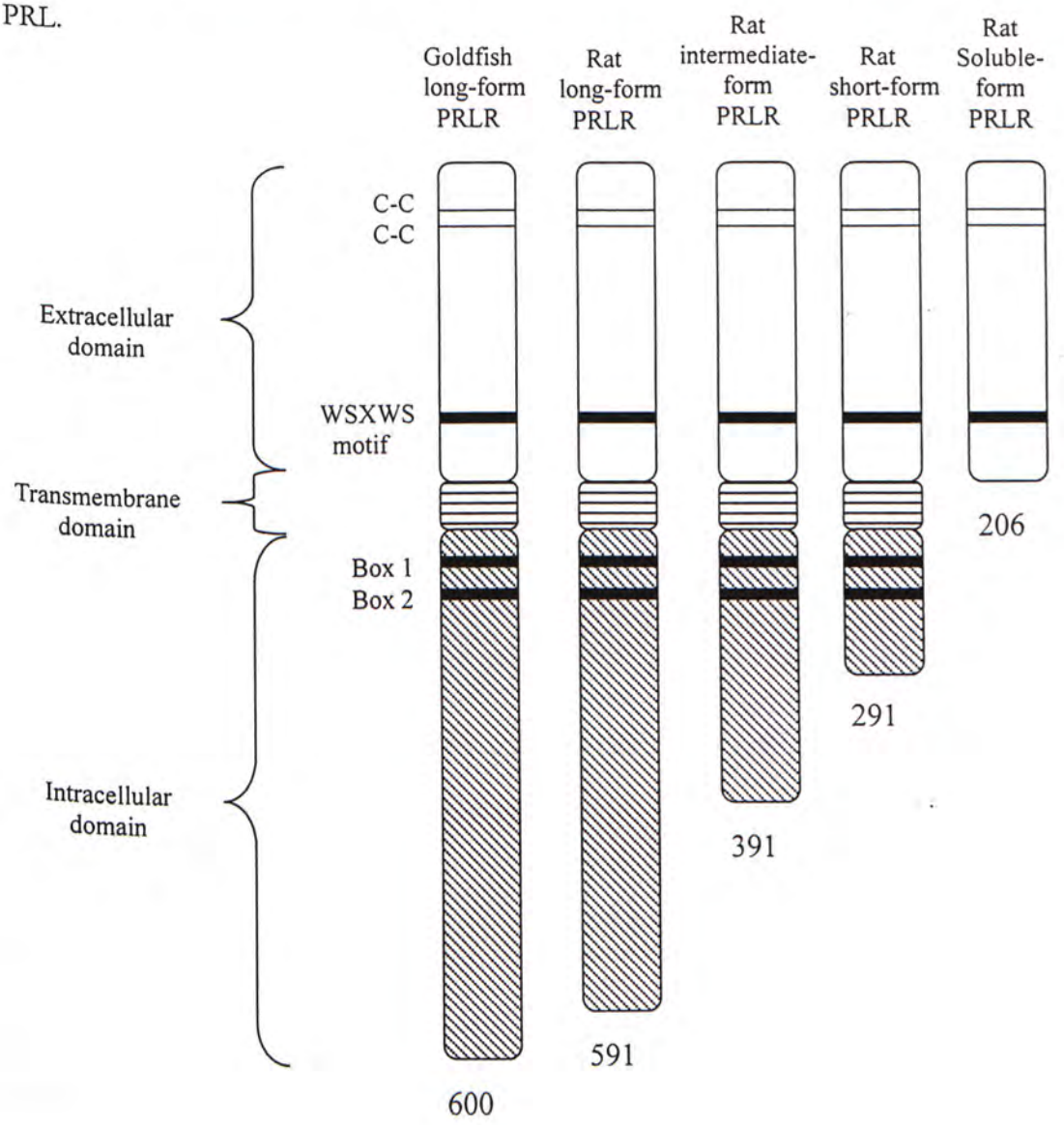


Fig.1.3. Schematic diagram of different forms of PRLR. Two cysteines pairs (C-C) and WSXWS motif located in the extracellular domain. Box 1 and Box 2 located in the intracellular domain. All are common characteristics of different forms of PRLR.

PRL receptors are present in nearly all tissues in the rat; the highest concentrations are in the liver, choroid plexus, ovary and mammary gland (Nagano and Kelly, 1994). It is likely that signal transduction by PRL requires binding of one PRL molecule to two receptors, similar to the case of GH molecules. The dimerization model, supported by X-ray crystallographic studies for GH (de Vos *et al.*, 1992), assumes that each PRL molecule has two binding sites that interact sequentially with the receptor (Rui *et al.*, 1994; Fuh *et al.*, 1993). At high concentrations, PRL can saturate the receptor and hinder further receptor dimerization, which explains the often-observed bell-shaped dose-dependent curves.

In some lymphocytes, PRL is also rapidly internalized and translocated to the nucleus (Rao *et al.*, 1993). Intranuclear accumulation of PRL is stimulated by interleukin-2 (IL-2), is maximal within 6 h of treatment, and is inhibited by extracellular anti-PRL antibodies (Clevenger *et al.*, 1990). However, the specific function of intranuclear PRL remains to be elucidated. There is evidence that in the choroid plexus (Walsh *et al.*, 1987), amniochorion (McCoshen and Barc, 1985) and mammary epithelial cells (Mercado and Baumann, 1994; Postel-Vinay *et al.*, 1991b), a PRL receptor/binding protein, possibly of a different structure, acts as a transporter that translocates PRL from blood to their respective fluid compartments, i.e. cerebrospinal fluid, amniotic fluid, and milk.

PRL signaling is mediated through a cytoplasmic tyrosine kinase pathway, a mechanism originally described for the interferon receptor (Darnell, Jr. *et al.*, 1994)(Fig. 1.4). The Janus tyrosine kinases (JAK) mediate signal transduction for most members of this superfamily. One of these, JAK2 is constitutively associated with the PRL receptor. Binding of PRL to its receptor causes phosphorylation of JAK2 and autophosphorylation of the distal intracellular domain of the receptor (Campbell *et al.*, 1994; Lebrun *et al.*, 1995). The activated JAK2 phosphorylates other associated proteins, most notably the Signal

Transducer and Activator of Transcription proteins (STAT). Of the STAT protein presently known, STAT 1, STAT 3 and STAT 5 are activated by PRL (Gouilleux *et al.*, 1995) in mammalian cells. The phosphorylated STAT proteins translocate to the nucleus where, probably in association with other proteins, they bind to specific responsive elements, called GAS ([Gamma]-interferon activated site), in the promoters of target genes and initiate their transcriptions. In T cells, there is evidence for an association between the PRL receptor and a serine theonine kinase RAF-1, although this kinase is not tyrosine phosphorylated by PRL as it is by other members of this superfamily (Clevenger *et al.*, 1994).

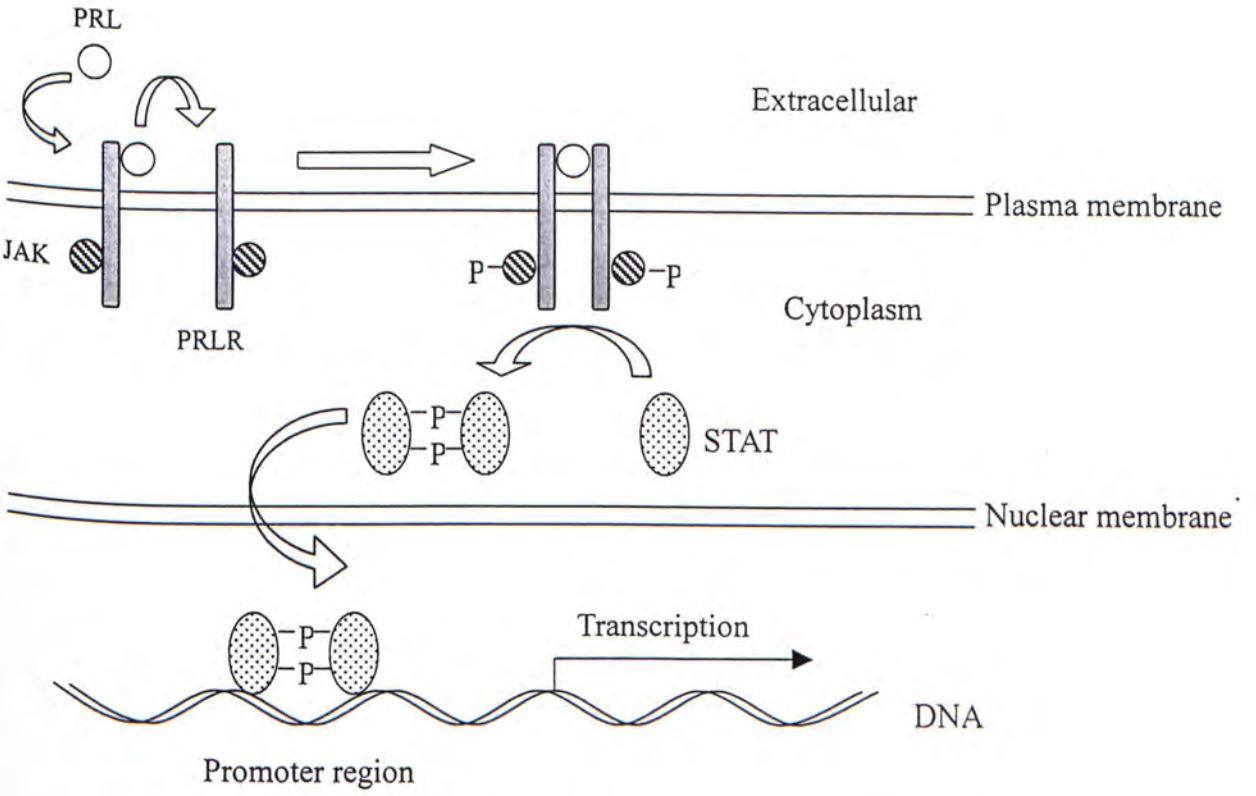


Fig.1.4. The signal transduction pathway of PRLR. "P" stands for phosphate which is introduced to the receptor and STAT by JAK tyrosine kinase (Groner B. *et al.*, 1995).

1.3 Biosynthesis of PRL

The mature PRL of about 23kDa is synthesized as a prehormone consisting of 277 aa in most mammalian species (Miller and Eberhardt, 1983). The mature hormone is derived from this precursor molecule by proteolytic cleavage of 28 aa signal peptide from the N-terminal(Lingappa *et al.*, 1977; Maurer *et al.*, 1977). PRL is mainly synthesized and secreted by lactotrophs of the anterior pituitary gland. However, PRL gene expression has been found in other tissues in mammals, including brain, decidua, myometrium, lacrimal gland, thymus, spleen, circulating lymphocytes, and lymphoid cells of bone marrow, mammary epithelia cells and tumor, skin fibroblasts, and sweat glands (Jonathan *et al.*, 1996).

In teleost, PRL is secreted by PRL cells located in rostral pars distalis of the pituitary gland. PRL gene expression at ectopic sites can be under regulatory mechanisms that are different from those for pituitary PRL gene; for example, in human decidual and lymphocytes PRL gene expression is under the control of an alternative promoter, not the pituitary promoter (Fig.1.5). The RNA transcript is approximately 150 bp larger than the pituitary counterpart (Gellersen *et al.*, 1989), containing an extra noncoding exon, exon 1a, at the 5'-untranslated region (UTR).

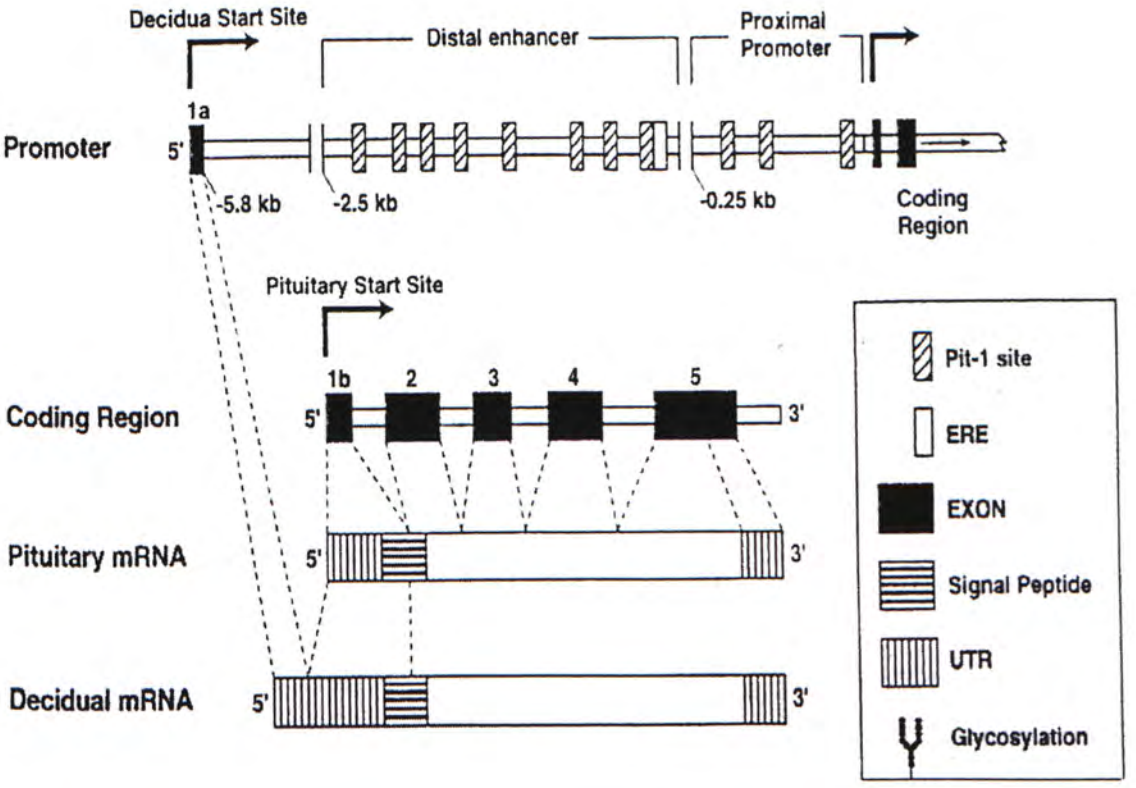


Fig.1.5. Diagram of the human PRL gene and mRNA. The PRL promoter has three regulatory regions:(1) a proximal region with three Pit-1 binding sites, (2) a distal region with eight Pit-1 binding sites, and an estrogen responsive element (ERE) sequence, and (3) a superdistal region (from –3.5 to 6kb). Extrapituitary sites (e.g. decidua) use an alternative promoter with the start site located 5.8kb upstream of the pituitary start site. The decidual-type mRNA is about 150bp longer in the 5'-UTR than the pituitary-type mRNA (Jonathan *et al.*, 1996).

1.4 Biological functions of PRL

PRL is a versatile hormone with more than 100 different actions, ranging from mammary development and lactation in mammals, to antimetamorphic effects in amphibians (Miller and Eberhardt, 1983).

One of the functions of PRL in teleosts is for osmoregulation, i.e. to maintain hydromineral balance of euryhaline teleosts in fresh water. In a marine environment, fish drink large amounts of water to replace water lost osmotically through the gills. They absorb ions from seawater and excrete them by active transport through the gills. Thus, marine fish can absorb water from the gut and replace what is lost. Fish excrete a minimal volume of urine (Nicoll, 1980). In fresh water, the body fluids are hypertonic to the external environment and they must cope with the problem of water inundation and the loss of ions that diffuse out through the gills. Adaptation to the freshwater habitat necessitates a reduction of gill permeability to salt loss and changes the transport process from active excretion to active uptake. The kidney eliminates the increased water by increasing the rate of urine excretion (Nicoll, 1980).

PRL is a fresh water-adapting hormone in regulating water and electrolyte balance through the gill and kidney. Chloride cells are the main ion-transporting cells of the gills where salt secretion occurs. These cells are rich in mitochondria for active uptake of ions. Chloride cells are responsible for Cl^- excretion in marine teleosts and for Cl^- absorption in fresh water teleosts. It should be noted that a passive Na^+ movement is always coupled to the active Cl^- transport (Day and Hinkle, 1988b; Day and Hinkle, 1988a; Foskett *et al.*, 1982). Na^+-K^+-ATPase is an ion-translocating enzyme present on the basolateral tubular membrane system of chloride cells (Philpott, 1980) to create ionic and electrical gradients for salt excretion. Ca^{2+}-ATPase is an enzyme located mainly in the plasma membrane of ionocytes

of the branchial epithelia. It takes up calcium ion directly from the freshwater via gills for growth and homeostasis (Flik *et al.*, 1985). To adapt freshwater environment, PRL increases the density of chloride cells to make their sizes become smaller with a lower level of Na^+-K^+-ATPase (Flik *et al.*, 1994; Herndon *et al.*, 1991) and hence resulted in the reduction of Na+ efflux. On the other hand, PRL enhances the calcium transport capacity of the gills by increasing the density of high-affinity Ca^{2+}-ATPase in plasma membrane of ionocytes, thus preventing the calcium efflux to water (Flik *et al.*, 1985; Flik *et al.*, 1989; Flik *et al.*, 1994).

Besides gills, PRL also exert its osmoregulatory effect on skin, intestine, kidney and urinary bladder. PRL stimulate mucus secretion at various body sites to reduce the skin permeability in order to reduce salt loss. In intestine, PRL decrease intestinal permeability to water and suppress the intestinal chloride pump (Morley *et al.*, 1981). In kidney, PRL increase Na+-K+-ATPase level so as to decrease the Na+ concentration in urine (Bonga, 1976; Wendelaar and Veenhuis, 1974). In the urinary bladder, PRL promotes the rate of reabsorption of filtered ions and decreases its permeability to water (Doneen, 1976). The osmoregulatory functions of PRL are summarized in Fig.1.6.

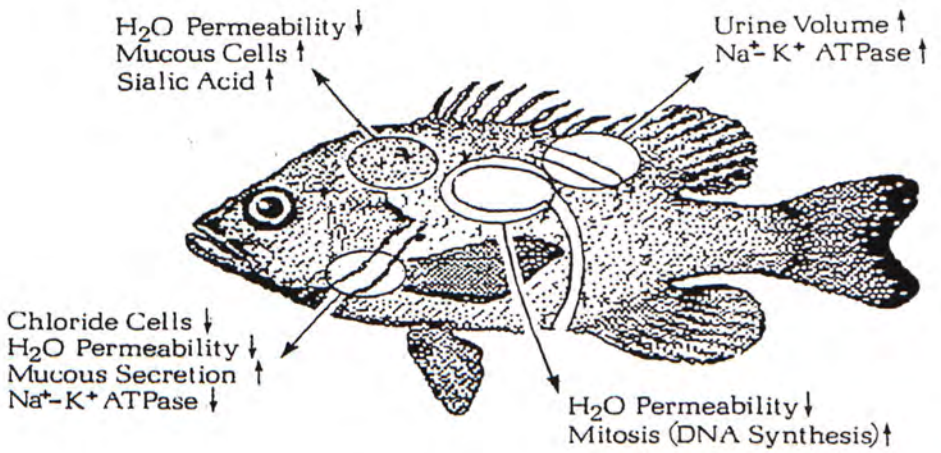


Fig.1.6 The major PRL targets and specific actions on teleost water and ion balance. The four major targets are (clockwise from upper left): the skin, kidney, urinary bladder, and gill (Adopted from Horseman 1987).

Other than osmoregulation, PRL also acts as a parental hormone. In female tilapia, PRL promotes brooding behavior of post-yolksac larvae (Weber and Grau, 1999). In male cichild fishes, PRL elicits fanning behavior, which provides freshwater to the eggs and as a result favor the egg development (de Ruiter *et al.*, 1986; Slijkhuis *et al.*, 1984).

In teleost, pigment movement is also regulated by PRL. Melanophores, erythophores and xanthophores are pigment cells that present in skin. Melanophores are for skin darkening and blanching. Erythophores and xanthophores are brightly pigmented cells. Dispersion of these two pigment cells can produce yellowish of reddish color skin. PRL can induce skin darkening in tilapia by forming pigment aggregation of melanophores (Kitta *et al.*, 1993). The effect was reversed after the removal of PRL (Kitta *et al.*, 1993). PRL can also cause pigment dispersion in tilapia (Oshima and Goto, 2000). The alteration of skin color enhances fish's ability to adapt to their living environment, such as escaping from their enemy.

1.5 Organization and regulation of the PRL gene

Mammalian PRLs are encoded by a single gene; in other words, at least only one has been detected so far. In teleost, there is evidence for more than one PRL gene (Chan *et al.*, 1996; Swennen *et al.*, 1992; Yamaguchi *et al.*, 1988; Yasuda *et al.*, 1986; Yasuda *et al.*, 1987). The PRL genes identified so far are made up of five exons and four intron(Miller and Eberhardt, 1983), but the human PRL gene (10kb in size) contains an extra noncoding, exon 1a for extrapituitary PRL expression(Ben Jonathan *et al.*, 1996; Frawley and Boockfor, 1991). All exon-intron boundaries, GT-AG, among rat, human and teleosts so far identified are strictly conserved. Teleostean PRLs, however, are smaller in size due to the smaller sizes of the introns.

PRL gene has two regions of promoter for its regulation: proximal promoter and distal enhancer regions. The proximal promoter region is located between –422 and +33 and the distal enhancer region is located between the coordinate –1831 and –1530 of rat PRL gene (Gutierrez-Hartmann *et al.*, 1987; Nelson *et al.*, 1986). These two regions were found in other animal species in similar locations. In human, a super distal promoter regions at –3500 to –5000 is identified and responsible for the regulation of PRL expression at extrapituitary sites as mentioned before (Berwaer *et al.*, 1994).

In the last decade, a number of studies have underlined the key role of the 5' flanking regions of the PRL gene in the dual control: tissue-specific and hormone-regulated expression. Nelson *et al* (1988) demonstrated the presence of four related cis-elements in both the distal enhancer (sites 1D to 4D) and in the proximal promoter regions of the rat PRL gene (sites 1P to 4P).

These nonadjacent multiple elements bind a common positive tissue-specific transcription factor which was cloned separately by two groups and referred to as Pit-1 or

GHF-1 (Bodner *et al.*, 1988; Ingraham *et al.*, 1988). This 31-33 kDa protein, hereafter termed Pit-1, exhibits a large 150-160 aa-long region of sequence similarity with three other transcription factors Oct-1, Oct-2, and unc-86 defined as the POU-domain proteins (Hérr *et al.*, 1988). The structure-function relationship of the Pit-1 protein was elucidated using mutational analyses (Elsholtz *et al.*, 1990; Ingraham *et al.*, 1990; Theill *et al.*, 1989). These studies revealed two regions in the POU-domain; a POU-homeodomain (aa residues 213-273) absolutely required for DNA binding, and a POU-specific domain (aa residues 128-198) which increases the affinity of Pit-1 binding for its consensus DNA sequence (A/TTATT/CCAT) and is involved in establishing correct DNA binding specificity (Fig.1.6).

The major trans-activating domain resides in the N-terminal region. The binding of Pit-1 to both the proximal and distal enhancer regions of the rat PRL gene as well as its role in conferring a characteristic pituitary phenotype to heterologous cell types was confirmed using recombinant Pit-1 factor (Mangalam *et al.*, 1989). While the proximal promoter is sufficient to confer tissue-specific expression to reporter genes transfected in rat pituitary cell lines (Lufkin *et al.*, 1989; Mangalam *et al.*, 1989; Nelson *et al.*, 1988), the distal enhancer/promoter element and the sequence flanking this enhancer are required to restrict expression to the lactotrope cells in the pituitary of transgenic mice (Crenshaw, III *et al.*, 1989).

A number of studies also suggested that Pit-1 was not in itself sufficient to account for the lactotrope phenotype, and that it was not restricted to this cell type. Not only Pit-1 transcriptionally activates the rat PRL gene, it also regulates the GH gene is also regulated by Pit-1 (Fox *et al.*, 1990; Ingraham *et al.*, 1988; Mangalam *et al.*, 1989). Second, repression of Pit-1 in somatic cell hybrids was correlated with the extinction of both rat PRL and GH

gene expressions (McCormick *et al.*, 1988; Supowit *et al.*, 1992). Analysis of anterior pituitary ontogeny by *in situ* hybridization and immunohistochemistry also showed that the Pit-1 protein was present in lactotropes, somatotropes and thyrotropes, but preceded the appearance of rat PRL and GH transcripts (Dolle *et al.*, 1990; Simmons *et al.*, 1990). Finally, the critical importance of Pit-1 for the expression of rat PRL and GH gene and in the survival of somatotropes, lactotropes and thyrotropes was illustrated by the discovery of Pit-1 genomic mutation in Snell and Jackson dwarf mice (Li *et al.*, 1990). Nevertheless, multiple subspecies of Pit-1 coexist in the pituitary gland and might lie at the root of the diverse cell phenotypes.

Several isoforms of Pit-1 have later been identified to be made from alternative splicing (Haugen *et al.*, 1993; Konzak and Moore, 1992; Morris *et al.*, 1992; Theill *et al.*, 1992)(Fig.1.7). Pit-1a, called also Pit-1 β or GHF-2, which contains an additional 26 aa in the trans-activation domain by alternative splicing at the end of intron one, was found as efficient as Pit-1 for trans-activating the GH promoter but did not trans-activate a rat PRL promoter-driven CAT construct. This suggested that the less abundant Pit-1 β is a more potent inducer of the GH promoter (Konzak and Moore, 1992). Indeed, Pit-1 β mRNA is present at a level of 14% of Pit-1 mRNA, and Pit-1 β protein is present at a level of less than 3% of Pit-1, so the significance of this variant in vivo is not clear at this time.

Another variant, Pit-1T, has been identified in thyrotroph-derived cells. It contains an insertion of 14 aa into the trans-activation domain. Pit-1T selectively stimulates the TSH-β promoter, but not the rat GH or rat PRL promoters in GH3 cells and hence suggested that the thyrotroph-specific Pit-1T exhibits a promoter-specific effect (Haugen *et al.*, 1994). A third variant, Δ4Pit-1, has been detected in all Pit-1 producing rat pituitary-derived cell

lines. Because of alternative RNA splicing, there is fusion of exon 3 to exon 5 with deletion of the POU-specific domain. Δ4Pit-1 cannot bind to the PRL first Pit-1 binding site of proximal promoter, 1P, nor can it transactivate the rat PRL promoter. But it can bind to other Pit-1 binding sites (Voss *et al.*, 1993). In gene transfer studies, Δ4Pit-1 inhibits PRL promoter activity, and therefore it may be potential mediator of PRL gene expression (Day and Day, 1994).

In teleost, a new isoform of Pit-1 was identified to contain an insertion of additional 33 aa (r-insert) in the N-terminal (Kausel *et al.*, 1999). This r-insert might have been lost during evolution in mammals, but its function is still unclear.

The reported Pit-1 alternative isoforms could participate in determining cell-specific expression of PRL gene. Post-translational modifications of Pit-1 might nevertheless improve tissue-specific activation of the rat PRL gene, e.g. phosphorylation at Thr-220 differentially affected the binding of Pit-1 to its target cis-elements in rat PRL and GH promoter (Kapiloff *et al.*, 1991). Another pituitary-specific transcription factor termed lactotroph-specific factor (LSF-1) that is distinct from Pit-1 was identified and purified from GH3 and GC cells by Gutierrez-Hartmann and colleagues (1987). However, LSF-1 cDNA has not been cloned and its primary structure remains unknown. Footprinting analysis also revealed that several LSF-1 binding sites overlap with the Pit-1 binding sites in rat PRL gene. Clustered point mutation analysis has demonstrated that altering sequence corresponding to any of the binding sites for Pit-1 considerably diminished the activity of the rat PRL promoter in transient transfection assays (Iverson *et al.*, 1990). Functional cooperativity between Pit-1 (d'Emden *et al.*, 1992) or LSF-1 binding sites is probably involved in rat PRL gene transcription.

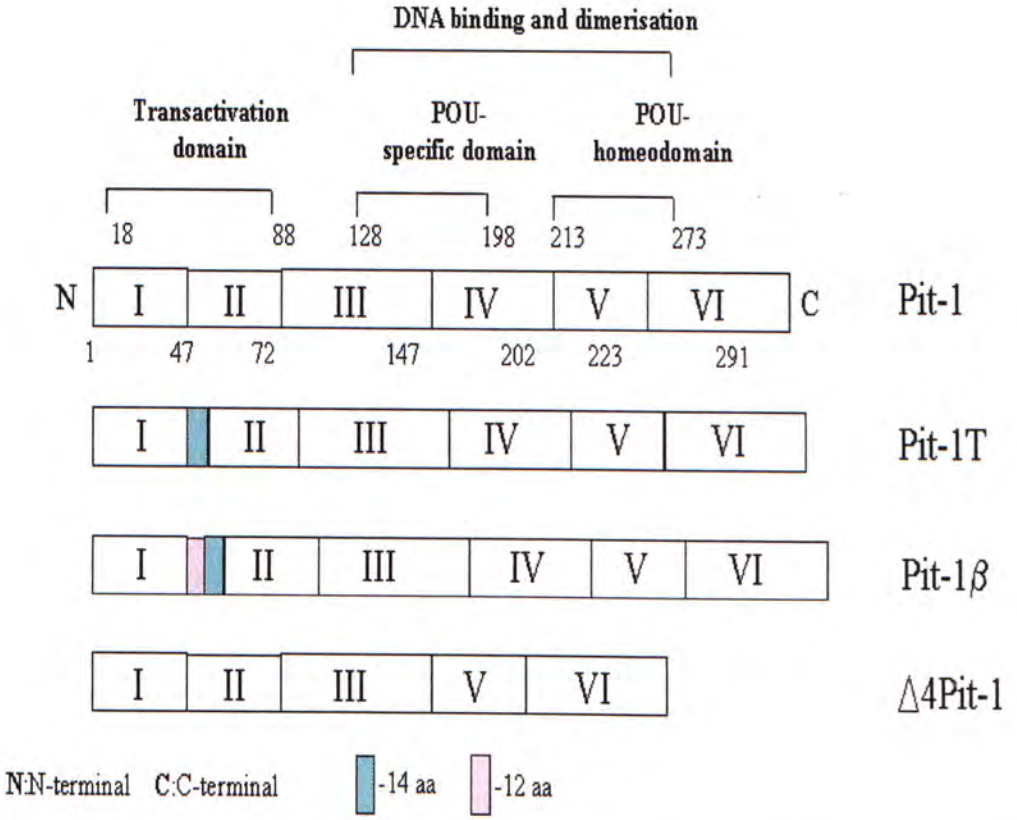


Fig.1.7. Pit-1 protein (six exons) and its splice variants. Pit-1T contains a 14 aa insert between exons 1 and 2. Pit-1b contains a 26 aa insert between exon 1 and exon 2. Pit-1 has a deletion of exon 4 (Cohen *et al.*, 1996).

Besides, LSF-1 is capable of activating the *in vitro* transcription and to bind multiple elements of the thyrotropin β-subunit promoter, suggesting that additional factors are required to restrict the expression of the rat PRL gene to lactotroph in the pituitary gland (Alexander *et al.*, 1990). These accompanying factors may be ubiquitous rather than tissue-specific. Coordinate actions of ubiquitous and tissue-specific transcription factors are required for specifying the lactotroph phenotype.

A number of studies indicated that protein/protein interaction constitute powerful mechanisms by which small number of factors acquires the ability to direct multiple although specific actions. Pit-1 mediates DNA-dependent protein-protein interaction. Pit-1 is monomeric in solution but associates as a dimer on its DNA response element (Ingraham *et al.*, 1990). In addition to the ability to form homodimeric complexes, Pit-1 can also associate with the widely expressed Oct-1 transcription factor (Voss *et al.*, 1991b). The resulting heterodimer binds on the most proximal binding Pit-1 binding site 1P of the rat PRL promoter and is functional, as shown by synergistic trans-activation of reporter constructs containing the 1P element in CV-1 cells co-transfected with Oct-1 and Pit-1 expression vectors (Voss *et al.*, 1991a). Such heteromeric complex could then play a role in the establishment of cell-specific gene expression, in as much Oct-1 is present early in development.

Other factors also act with tissue-specific factors but via at least partially distinct cis-element. Such an element, localized between the 1P and 2P sites at position –97 to 71, contains sequences with similarity to cAMP response elements and to the recognition site for transcription factor AP2. Linker scanning mutagenesis for this element had substantial inhibitory effect on basal promoter activity in both pituitary and non-pituitary cell lines co-transfected with Pit-1 expression vector (Iverson *et al.*, 1990). Further studies involving

site-specific deletion, heterologous gene promoter assay and gel shift analyses have indeed permitted the delineation of a basal transcription element (BTE) which resides between position –112 to –85 (Jackson *et al.*, 1992). This region binds a basic transcription factor (BTF) that trans-activates the rat PRL promoter in pituitary cells.

It is well established that steroid and steroid-like hormone receptors are transcription factors and thereby regulate rat PRL gene transcription (Davis *et al.*, 1988; Rosenfeld *et al.*, 1987). Estrogen receptor is one of these transcription factors that has been extensively investigated. The ligand-activated estrogen receptor is an important co-factor of tissue-specific trans-activators. A sequence conferring response to estrogen responsive element (ERE) is present in the distal enhancer region (Day and Maurer, 1989; Maurer and Notides, 1987; Waterman *et al.*, 1988). This non-palindromic ERE sequence resides within position –1581 to –1569, and partially overlaps the 1D Pit-1 binding site. Co-transfection of non-pituitary CV1 cells (Simmons *et al.*, 1990) or Rat-1 cells (Day *et al.*, 1990) with expression vectors for Pit-1 and the estrogen receptor indicated that maximal expression of reporter genes under the control of the distal enhancer and promoter proximal regions required both Pit-1 and the estrogen receptor. Similarly to Oct-1, the estrogen receptor is present early in development and was thus proposed as controlling factor in the ontogeny of rat PRL gene expression. The ability of estrogen to participate in the specification of the lactotrope phenotype is of particular interest when considering that this steroid was formerly identified as the major rat PRL stimulating hormone (Davis *et al.*, 1988; Maurer, 1982; Rosenfeld *et al.*, 1987). This already indicated that hormone-dependent and tissue-specific regulations are intricated.

In addition, the rat PRL gene promoter is also the ultimate target of intracellular signaling pathways activated at the plasma membrane by classical hypophysiotropic peptides

and ubiquitous growth factors. Thyrotropin-releasing hormone (TRH) was the first peptide shown to increase rat PRL gene transcription (Davis *et al.*, 1988; Murdoch *et al.*, 1983). TRH acts via a receptor of the seven transmembrane-domains family linked to trimeric GTP-binding protein ($G_{aq/a11}$) that activates the phosphatidylinostitol cascade and ensuing increase in cytosolic Ca_i^{2+} asctivation of protein kinase C (PKC) and influx of calcium (Hsieh and Martin, 1992; Zhao *et al.*, 1992). Yan (1991) thereafter demonstrated by transfection studies that the activation of rat PRL promoter by TRH is Pit-1 dependent.

As far as inhibitory regulations of the rat PRL gene expression are concerned, the major physiological regulator is the neurotransmitter DA. The ability of DA to inhibit the transcription of the rat PRL gene was actually the first demonstration of a transcriptional regulation of the rat PRL gene (Maurer, 1981). These results, obtained using normal cells, were confirmed and further documented when GH cells stably transfected with a D2 receptor expression vector became available (Albert *et al.*, 1990). Using this model, Elsohltz et al. (1991) were able to show that DA or the DA agonist bromocryptine strongly inhibited the activity of a reporter gene driven by the proximal promoter region and which is Pit-1 dependent. Many studies suggested that Pit-1 functions as a cell-specific signal integrator of different hormone stimulation (Bradford *et al.*, 1997) (Fig.1.8).

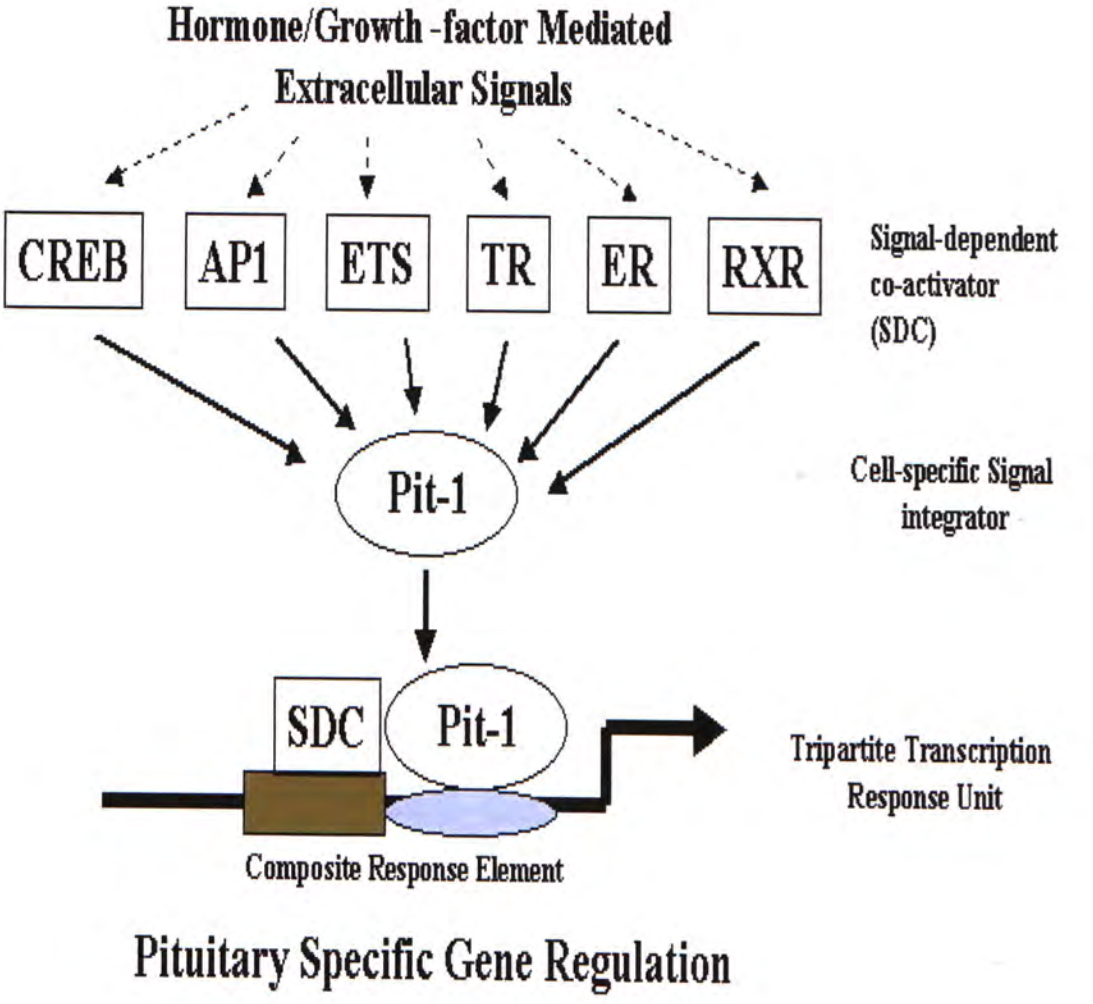


Fig.1.8. Model for pituitary-specific signal integration by homeodomain protein GHF-1/Pit-1. Extracellular signals are targeted to nuclear co-activators such as cAMP-responsive element binding protein (CREB), the Jun/Fos family (AP-1), members of the Ets family of transcription factors (ETS), thyroid receptor (TR), estrogen receptor (ER), or retinoid receptor (RXR). Such inductive signals alter the transactivation potential of these co-activators. Functional interaction of these signal-dependent co-activators (SDCs), e.g. Ets1, with Pit-1, at a composite Pit-1/Co-activator DNA binding site, forms a tripartite response unit, which permits highly specific pituitary transcriptional responses to general signaling pathways. Thus, Pit-1 functions as a cell-specific nuclear integrator of diverse extracellular hormone/growth factor signals (Bradford *et al.*, 1997).

1.6 Aims of this study

GH and PRL belong to the same family of polypeptide hormones and they have subjected to extensive investigation in teleost because of their osmoregulatory function. The major roles of both hormones in fish are very different from that in mammals, GH is for seawater adaptation while PRL is for fresh water adaptation. They show an inverse relationship which attracts the attention of scientists. Their roles were clearly demonstrated in euryhaline teleost such as tilapia as they migrate from seawater to freshwater and vice versa. However, what happens to a fish which always live in a freshwater? Does the hormone level remain unchanged to correspond to the relative constant osmotic environment? If not, what contribute to the changes? We would like to understand more about the aspect of freshwater teleost endocrine system, using goldfish as a model to study:

1. the genomic structure of gfPRL,
2. the hormonal regulation of gfPRL expression,
3. the environmental regulation of goldfish hormones expression, and
4. the interaction between gfPRL and its receptor.

Chapter Two

PCR Cloning of gfPRL Gene

2.1 Introduction

PRL is a member of the polypeptide family of hormones that includes GH, somatolactin (SL) and placental lactogen (PL). This family is believed to have evolved from a single ancestral gene by gene duplication and sequence divergence (Horseman and Yu-Lee, 1994; Niall *et al.* 1971; Chen *et al.*, 1994). To understand the regulatory mechanism of teleost PRL expression in response to osmotic potential, PRL genes from tilapia and trout were identified to study its regulation at molecular level. However, most of the investigations were focused on euryhaline teleosts or migrating fish species in order to study the roles of PRLs in osmoregulation. Little is known about PRL gene regulation in fresh water teleost. Goldfish is a fresh water teleost, it provides an excellent model to investigate the role of PRL in fresh water fish. Moreover, understanding of the gene sequence of PRL in goldfish provides a platform to further our investigation on the PRL gene regulation.

This chapter describes the use of genomic PCR method to obtain a gene fragment of goldfish PRL with a regulatory region carrying 8 putative Pit 1 binding sites and a TATA box.

2.2 Materials and Methods

2.2.1 Buffers and Reagents

Acrylamide Solution, 30:8, 40% (w/v)

Acrylamide	30g
Bis-acrylamide	0.8g
Distilled water	mix up to 100ml

Filter with 3MM Whatmman paper
Store at 4°C temperature.

Bromophenol blue, 10% (w/v)

Bromophenol blue	1g
Distilled water	mix up to 10ml

Store at room temperature.

Denaturing Solution

NaCl	(1.5M) 87.66g
NaOH	(0.5M) 20g
Distilled Water	mix up to 1L

Store at room temperature

Denhardt's solution, 100 x

Polyvinylpyrrolidone (PVP)	10g
Bovine serum albumin (BSA)	10g
Ficoll 400	10g
Distilled water	460ml

Store at -20°C in 50ml aliquots.

Ethidium bromide (EtBr), 10mg/ml

Ethidium bromide	0.2g
Distilled water	mix up to 20ml

Store at room temperature and prevent light exposure

Ethylenediamine tetraacetic acid (EDTA), 0.5M

$Na_2EDTA \bullet 2H_2O$	93.05g
Distilled water	mix up to 300ml
Adjust the pH to 8.0 with 10M NaOH and Distill water	mix up to 500ml

Autoclave and store at room temperature.

Hybridization buffer for Southern blot analysis

100x Denhardt's Solution	5ml
20x SSC	30ml
10% SDS	5ml
Distilled water	mix up to 10ml

Freshly prepare before use

Hybridization solution for primer extension, 5X

NaCl	(2M) 11.68g
PIPES	(40mM) 1.21g
$Na_2EDTA \bullet 2H_2O$	(10mM) 0.3722g

Adjust to pH 6.8

Distilled water	mix up to 100ml

Store at room temperature

Isopropyl-β-D-thiogalactopyranoside (IPTG), 100mM

IPTG	238mg
Distilled water	mix up to 10ml

Filter with 0.22μm membrane

Store in 1ml aliquots at -20°C.

Loading dye, 6X

Sucrose	4g
Bromophenol blue	0.025g
Xylene cyanol	0.025g
Distilled water	mix up to 10ml

Store at 4°C.

Neutralization Solution

NaCl	(1.5M) 87.66g
Tris Base	(0.4M) 48.4g
Distilled Water	mix up to 1L

RF1 Solution

Potassium Acetate	30mM
$CaCl_2$	10mM
$MnCl_2$	10mM
Glycerol	15%

RF2 Solution

MOPS, pH 6.5	10mM
$CaCl_2$	75mM
Glycerol	15%

Adjust to pH 6.5 by KOH.

Sodium acetate, 3M

Sodium acetate•$3H_2O$	40.8g
Distilled water	80ml
Adjust pH with glacial acetic acid	
Distill water	mix up to 100ml

Autoclave and store at room temperature.

Sodium dodecyl sulfate (SDS), 10% (w/v)

SDS	100g
Distilled water	800ml
Heat to 50°C to dissolve.	
Distilled water	mix up to 1L

Store at room temperature

SSC buffer, 20X

NaCl	(3M) 175.3g
Trisodium citrate	(0.3M) 88.2g
Distilled waer	800ml
Adjust the pH to 7.0 by 10M NaOH.	
Distilled water	mix up to 1L

Autoclave and store at room temperature

TAE buffer, 50X

Tris base	(2M) 242g
Sodium acetate	(1M) 136.1g
Na_2EDTA	(50mM) 19g
Distilled water	700ml
Adjust pH to 7.2 with acetic acid.	
Distilled water	mix up to 1L

Autoclave and store at room temperature

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 20mg/ml

X-gal	20mg
Dimethylformamide (DMF)	1ml

Store at -20°C in glass container.

2.2.2 Methods

2.2.2.1 PCR of the 5' flanking region of gfPRL gene

The PCR reaction was preformed following the instructions of Genome Walker Kit (CloneTech) manual (Fig. 2.2A). The gene specific primers used were gfPRLR2 and gfPRLR3 (Table 2.1 and Fig. 2.1). The first PCR, using AP1 and gfPRLR2 primers, was carried out under the following conditions

94 ℃ 2 min

94 ℃ 1 min
55 ℃ 1 min
72 ℃ 3 min
} 30 cycles

72 ℃ 10 min

The PCR products were 10-fold diluted and used as a template for second PCR, using AP2 and gfPRLR3 as primers, and carried out under the following conditions.

94 ℃ 2 min

94 ℃ 1 min
55 ℃ 1 min
72 ℃ 3 min
} 30 cycles

72 ℃ 10 min

For each PCR, 0.5 unit of the enzyme (Expand^{Tm} High Fidelity PCR System) (Roche) was used in a 50 µl reaction mixture. All reactions were carried out on the Stratagene Robot Cycler 9600.

2.2.2.2 Genomic PCR of gfPRL gene

The genomic PCR was carried out using gfPRLF1 (10 pmoles) and gfGCR (10 pmoles) as primers. Goldfish genomic DNA (2 μg) was used as template. Expand^{Tm} High Fidelity PCR System (0.5 unit enzymes; Roche) was used to amplify the gene in a 50 μl reaction mixture. The PCR condition was as followed:

94 ℃ 2 min

94 ℃ 1 min
55 ℃ 2 min
72 ℃ 3 min
} 30 cycles

72 ℃ 10 min

The reaction was carried out on a Perkin Elmer 9600.

2.2.2.3 Spectrophotometric analyses of DNA and RNA

The amount of nucleic acids was determined at 260 nm by using a Shimadzu UV1601 spectrophotometer. Five μl of sample was mixed with 995 μl distill water (200-fold dilution). One unit approximately equals to 10 μg/μl dsDNA. On the other hand, Four microliters RNA sample was mixed with 996 μl distill water (250-fold dilution) and 1 unit corresponds to 10 μg/μl RNA. The purity of the nucleic acid preparation was determined by the ratio between the readings at 260nm and 280nm. High purity of DNA should have ratio of 1.6 or above, while high purity of RNA should has a ratio of around 2.0.

2.2.2.4 Agarose gel electrophoresis of DNA

DNA fragments were resolved by 1% (w/v) agarose gel in 1X TAE buffer. The agarose gel was pre-stained by adding ethidium bromide (EtBr) in the gel. One volume of 6X loading dye was mixed with 5 volumes of the DNA sample. The DNA sample was loaded

into the gel and run at 100V. The gel was examined under ultraviolet (UV) trans-illumination and recorded using a UVP gel documentation instrument.

2.2.2.5 DNA radioactive labeling by random priming

The radioactive probe for Southern hybridization was labeled with ^{32}P by using the Nick Translation Kit N500 (Amersham Pharmacia Biotech). This protocol was modified from the supplier's suggested method. Around 1-5μg of purified DNA probe (1kb DNA fragment of gfPRL P1A cDNA which was released by restriction enzymes digestion from cDNA clone) was mixed with 10 μl of nucleotide mix, 5 μl of α-^{32}P-dCTP (25 μCi/μl), 5μl of enzyme mix and the final volume was adjusted to 50 μl. The mixture was incubated in a 14-15 °C water bath for 2 h. Radioactive labelled DNA probe was purified using the MicroSpin column to remove the unincorporated radioactive nucleotide. The radioactively labelled DNA fragment (~200,000 cpm after spin-column, incorporation rate was around 50%) was stored at –20 °C.

2.2.2.6 Vacuum transfer of DNA fragments onto a nylon membrane

The agarose gel was denaturated in denaturing solution for 15 min. It was rinsed with distilled water and then neutralized in neutralization solution for 15 min twice. The gel was ready for DNA transfer. A nylon membrane of the same size as the gel was placed on the blotter first while other exposed area was covered with paraffin. As a result, the nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) was the only air passage. The treated gel was carefully placed on the nylon membrane without air bubble trapped inside. 20X SSC solution was poured on the top of the gel and covered all the area, and then the vacuum pump was turned on to start the vacuum transfer. The pressure was kept at 50 mbar, and the transfer was lasted for 1.5 to 2 h with continues supply of 20X SSC until the DNA

was completely transferred to the blot. The DNA on the nylon membrane was cross-linked with UV in an UV cross-linker (DNA Tranfer Lamp, Fotodyne) for 4 min.

2.2.2.7. Southern blot analysis

The nylon membrane was soaked in 50 ml hybridization buffer with calf thymus DNA (200 µl) for 2 h at 65 °C. The calf thymus DNA was denatured at 99 °C for 5 min, ice-chilled and used as blocking agent for non-specific hybridization. The radioactive-labelled probe was then added and the membrane was incubated with the probe overnight at 65 °C. Finally, the hybridized membrane was collected and washed twice with 1XSSC 10% SDS, 1X SCC 1% SDS, and 1X SSC 0.1% SDS sequentially. After washing, the membrane was wrapped for Molecular Imager (BioRad GS505) analysis.

2.2.2.8 Molecular Imager Analysis

Molecular Imager (Bio-Rad GS-505) was used to detect radioactive signals from paper/membrane sources. It mainly consists of three components: radioactivity sensitive screen, scanner and eraser. The scanner was turned on for at least 15 min before use (warming up process). The screen was put into the eraser for at least 10 min to remove any residual signals.

The wrapped hot membrane was leaved on a platform, called Load Dock Exposure Pad, before making a sandwich with a clean screen. The membrane was fixed with tapes and covered by an aluminum-like foil of Screen-Guard film. Then the pad was placed into the Loading Dock in where the membrane was sandwiched by the clean screen. The screen began to record the signals from the membrane once they contacted each other closely. After 10 min (typically 1/10 the exposure time for film), the screen was separated from the membrane in the Loading Dock and was inserted into the scanner to record the signals. The recorded signals were analyzed by using a computer software, Molecular Analyst.

2.2.2.8 Phosphorylation of PCR amplified DNA

The DNA fragment of interest in the gel was purified with Concert™ Rapid Gel Extraction System (GibcoBRL). The purified DNA fragment was subjected to phosphorylation for ligation into plasmid vectors. The purified DNA was added with 10 µl of 5X Forward Reaction Buffer (GibcoBRL), 1µl of 10mM dNTPs, 1µl of 0.1M ATP, 10 units of T4 polynucleotide kinase (Promega) and sterile distilled water to a final volume of 100µl; and the reaction mixture was incubated at 37 °C for 1 h. Then the modified DNA was further purified by Concert™ Rapid PCR Purification System (GibcoBRL) and stored at –20 °C.

2.2.2.9 Ligation of DNA fragment to linearized vector

The purified DNA fragment was ligated to the linearized vector, pBluescript II SK (+/-) (Stratagene). The vector (0.1 µg), which was linearized by *Sma* I, and 0.6 µg DNA (2:1 to 3:1 in DNA to vector ratio) were mixed together. Two microliters 10X ligation buffer (GibcoBRL) and 1 unit of T4 DNA ligase (GibcoBRL) were added. Sterile distill water was added to a final volume of 20 µl. The mixture was incubated for 16 h at 16 °C. The mixture was stored at –20 °C and ready for transformation.

2.2.2.10 Preparation of *Escherichia coli* competent cells

Escherichia coli (E. coli) strain DH5α was streaked on the surface of a LB (Luria-Bertani) agar plate and incubated for 16 h at 37°C. A single colony was transferred to 5 ml LB medium and incubated at 37 °C for 2 h. Then this 5ml LB medium was inoculated into 100ml LB medium. The cells was grown at 37 °C until OD_{600} reached 0.4 - 0.5, and was immediately chilled on ice for 150 min The cells were harvested by centrifugation at 1,000X g for 15 min at 4 °C and further resuspended in 37 ml RF1 and kept on ice for 15 min The

cells were centrifuged again at 1,000g for 15 min at 4°C. The cell pellet was resuspended by 8 ml RF2 and kept in ice for 15 min The competent cell suspension was dispensed into aliquots of 100μl each and immediately frozen in liquid nitrogen. The frozen competent cells were stored at -70°C and could be thawed immediately before use.

2.2.2.11 Bacterial transformation by heat stock

The plasmid DNA after ligation to the DNA fragments was transformed into DH5α competent cells. Half of ligation mixture (10 μl) was added to 100 μl competent cells, and kept on ice for 30 min. The cells and plasmid was heat-shocked at 42 °C for 1.5 min and ice-chilled immediately. LB medium (0.9 ml) was added to the mixture, and incubated at 37°C for 1 h. The cells were harvested by centrifugation (12,000X g for 1 min). It was spread on a LB plate containing ampicillin (1 μg/ml) alongside with 20 μl (20 mg/ml) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 20 μl 0.1M Isopropyl-β-D-thiogalactopyranoside (IPTG). The plate was incubated for 16 h at 37 °C.

2.2.2.12 Automated PCR sequencing

Large amount of high purity plasmid containing the insert of our interests was prepared using Concert™ Rapid Maxi Prep Purification System (GibcoBRL). PCR sequencing was performed using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The double-stranded DNA in 200-500ng (per 1 kb in size) was acted as the template of the reactions. Then 3.2 pmole of primer and 8μl of Terminator Ready Reaction Mix were added into the tube with the template. Sterile distilled water was added to a final volume of 20 μl. The reaction was preformed in a GeneAmp PCR System 9600 (Perkin Elmer) and the conditions were as follows.

end-labeled primer (>1x10^5 cpm) in a total of 30 µl 1X hybridization buffer, incubated at 95°C for 10 min, and immediately transferred to 56°C for 16h. Reagents were added directly to the hybridization mixture for a total volume of 50 µl 1X reverse transcriptase buffer containing 10mM dNTP, 5 units RNaseOut (GibcoBRL), and 200 units Superscript reverse transcriptase (GibcoBRL).

The primer extension reaction was incubated at 37°C for 1 h. RNA was subsequently digested with 12.5 µg RNase A (Amersham Pharmacia Biotech) at 37°C for 30 min, followed by ethanol precipitation. The pellet was dissolved in 5 µl distilled water and ready for polyacrylamide gel electrophoresis.

The sequencing reaction was also preformed using T7 Sequencing Kit (Amersham Pharmacia Biotech) for comparison to identify the exact position of the extension ends. The sequencing procedures was followed the instructions of the kit manual with the primer used for the primer extension and the cloned PRL gene fragment as template. The sequencing products and the reverse transcription product were analyzed together on a 8% polyacrylamide urea gel in different lanes.

96°C	10 sec	25 cycles
55°C	5 sec	
60°C	4 min	

To ensure that the reaction was completely terminated, 1 μl calf intestine alkaline phosphatase (CIAP) (Promega) was added to the reaction mixture for 30 min at 37 °C. After dephosphylation process, the DNA content of the reaction mixture was precipitated by 50 μl absolute ethanol with 2 μl 3M pH4.6 sodium acetate. The precipitation process was held at –20 °C for at least 2 h and the solution was centrifuged (Eppendorf 5417R) at 12,000X g for 30 min at 4 °C. Then the supernatant was removed carefully and the the DNA pellet was rinsed with 250μl of 70% ethanol, and centrifuged again at 12,000X g for 5 min The supernatant was discarded, and the pellet was dried in a vacuum centrifuge (Speed Vac) for 2 min. The dried pellet was resuspended in 12μl of Template Suppression Reagent (TSR) and denatured at 95°C for 2 min and ice-chilled immediately. The DNA sample was ran in ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) and the results were analyzed by the ABI sequence analyzer with a Macintosh software.

2.2.2.13 Primer extension using reverse transcription

A synthetic oligonucleotide primer, gfPRLR3, of 50 pmoles was end-labelled with 2ul γ-32P-ATP (250 μCi/ul; Amersham Pharmacia Biotech) and 5 units of polynucleotide kinase (GibcoBRL) in a 30 μl reaction mixture at 37°C for 30 min Afterwards, 50 ul absolute ethanol and 3 μl 3M sodium acetate were added to precipitate the labelled DNA.

Pituitary total RNA of goldfish (25-30 μg) was mixed with approximately 50 pmoles 5'

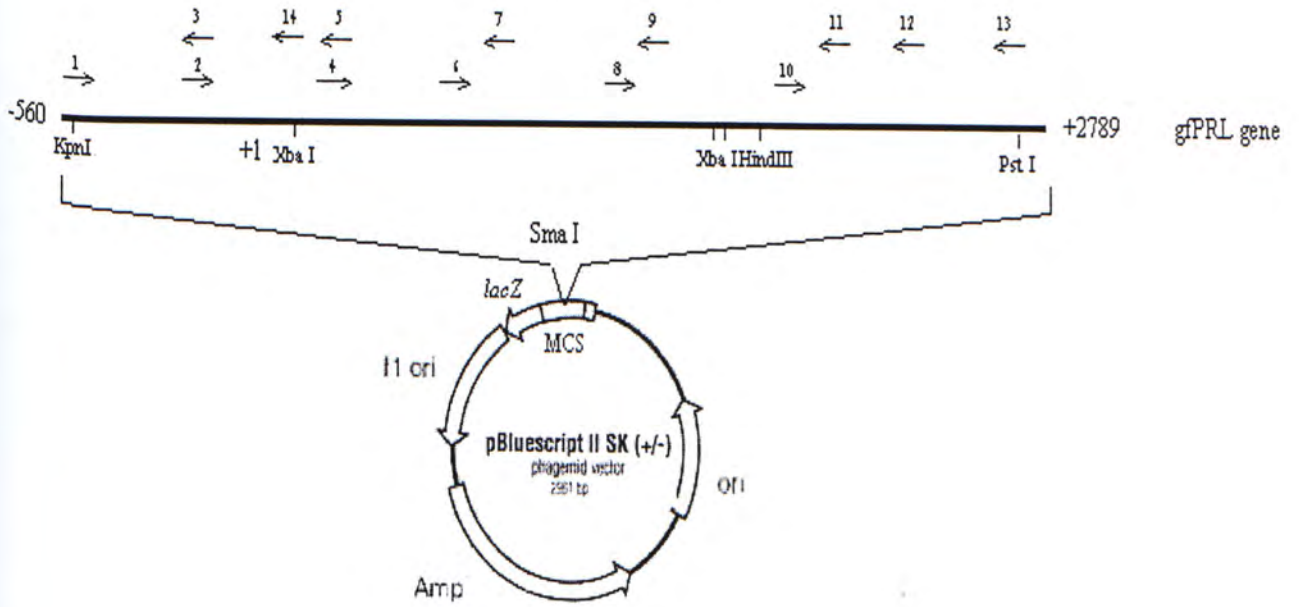
2.3 Results

2.3.1 Cloning of the 5' flanking region of gfPRL gene

The first round PCR products using PRLR2 (reverse primer) and AP1 (forward primer) (Fig. 2.1 and Table 2.1) were resolved by agarose gel electrophoresis and visualized by transillluminator after EtBr staining. Only smear products were obtained in the first round PCR as shown in Fig. 2.2B. Nested primers PRLR3 (reverse primer) and AP2 (forward primer) (Fig. 2.1 and Table 2.1) were further used in the second round PCR using the first round PCR products as template. Again, the products were resolved by agarose gel electrophoresis and visualized by transilluminator after EtBr staining (Fig. 2.2C). Sharp and discrete single bands were obtained after nest-PCR in lane 2 (*Dra* I digested genomic DNA after ligation to linkers as templates, ~50bp), lane 3 (*Stu* I digested genomic DNA after ligation to linkers as templates, ~200bp), and lane 5 (*Pvu* II digested genomic DNA after ligation to linkers as templates, ~600bp) respectively. The ~600bp PCR product was isolated using Concert Rapid Gel Isolation Kit and cloned into pGem-T easy vector (Promega). The complete sequence of this PCR product is shown in Fig. .2.3.

Table 2.1 Nucleotide sequence of oligonucleotide primers used in gene se

Number	Primer name	Nucleotide sequence
1	gfPRLF3	5' GCGGTACCTACAATATATTTAG 3'
2	gfPRLF2	5' GGCACACAGCTCATTAAATGTCAC 3'
3	gfPRLR5	5' GTGACATTTAATGAGCTGTGTGCC 3'
4	gfPRLF15	5' GTGTGGGTTTGTCTCCATC 3'
5	gfPRLR2	5' ACCGTTGATGGAGACAAACCCACACAT
6	gfPRLF12	5' CCCTGTAACAACTTCCGTAC 3'
7	gfPRLR14	5' GCTGGAACACACTCTCAG 3'
8	gfPRLF13	5' GCCTTGTGTATAAATAGG 3'
9	gfPRLR17	5' GTGCCAGGCTGGACGCCTC 3'
10	gfPRLF17	5' TACCATTTCCATGGTGAAG 3'
11	gfPRLR19	5' AACATGGTCTGATGGTCTC 3'
12	gfPRLR13	5' GATGTCAAAAGGGAGAGAGG 3'
13	gfGCR	5' GCAGAGCACAGCATTTCCT 3'
14	gfPRLR3	5' CAGTCTAGATCCTTGAGTCATT 3'



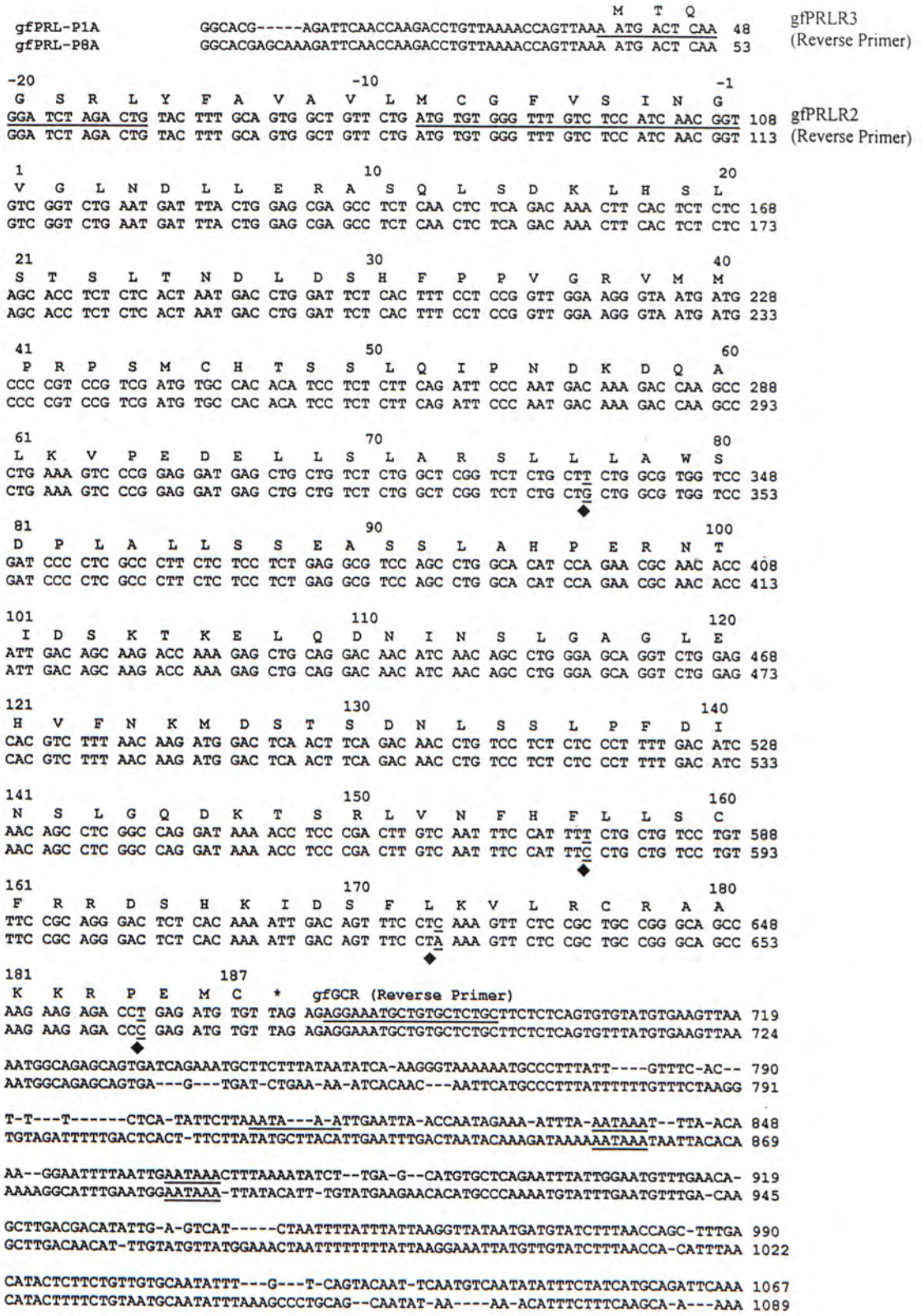


Fig.2.1. Nucleotide sequences of gfPRL cDNAs. The underlined sequences are the location of gfPRLR2 and gfPRLR3 used for Genome Walker PCR (Chan *et al.*, 1996).

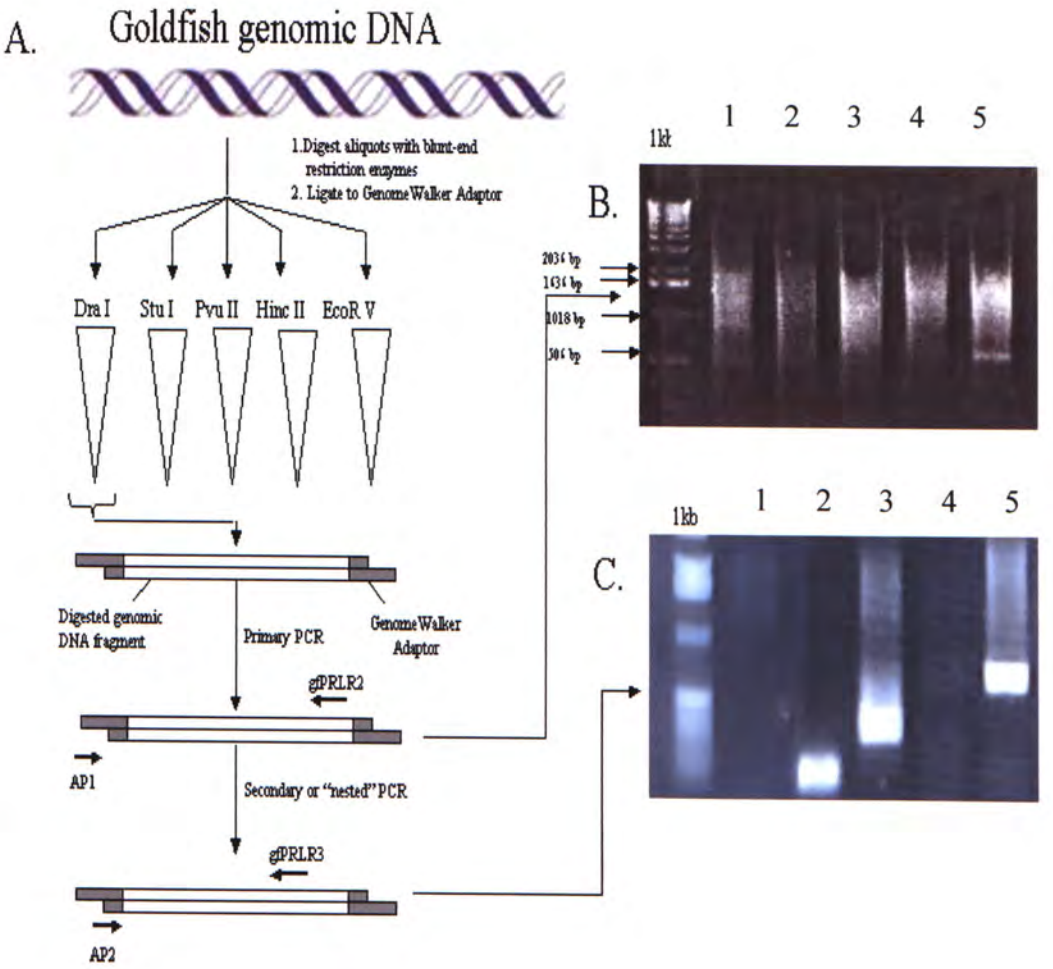


Fig.2.2. Amplicons of gfPRL gene fragments. A. Schematic diagram of the strategy of the Genome Walker Method. B. Primary (first round) results of genomic PCR using AP 1 and gfPRLR2 primers. C. Second round or "nested" PCR results using nested primers, AP1 and gfPRLR3. Lane 1: Hinc II digestion; Lane 2: Dra I digestion; Lane 3: Stu I digestion; Lane 4: EcoR V digestion; Lan5: Pvu II digestion.

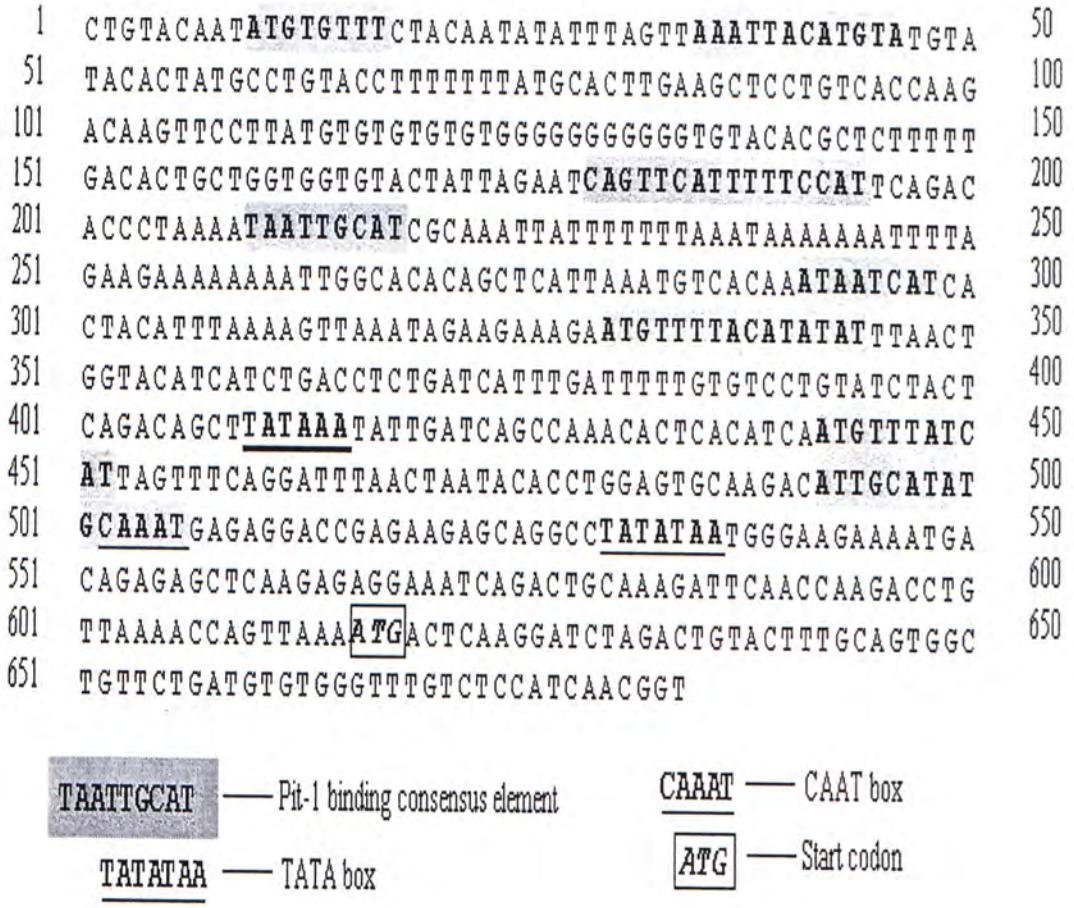


Fig.2.3. Nucleotide sequence of the 5' flanking region of the gfPRL gene. The DNA fragment from Genome Walker Kit (Pvu II) was cloned into pGem-T-easy vector and sequenced. It contains 684 nucleotide (including the partial sequence of the first exon). The letters highlighted with gray is the putative *Pit*-1 binding sites. The bolded and underlined letters represents the TATA box and CAT box. The italic letters with hollow frame showed the start codon.

2.3.2 PCR cloning of gfPRL gene

Gene specific primers gfPRLF3 (forward primer) and gfPRLGCR (reverse primer) were designed at the positions shown in Table 2.1. A single discrete band of a size of ~3.2kb was amplified. In order to confirm its identifty, it was transferred to nylon membrane and subjected to Southern hybridization using radioactive-labelled gfPRL P1A cDNA as a probe. The P1A cDNA itself acted as a positive control. The fragment was hybridized with the radioactive-labelled probe as strong as the positive control (Fig. 2.4) suggesting that the fragment contained gfPRL cDNA sequence. It was cloned into pBluescript vector and its nucleotide sequence determined by automatic DNA sequencing as described in the Materials and Method section. The sequencing strategy and restriction map is shown in Table 2.1. The entire sequence of gfPRL gene P1A is shown in Fig. 2.5.

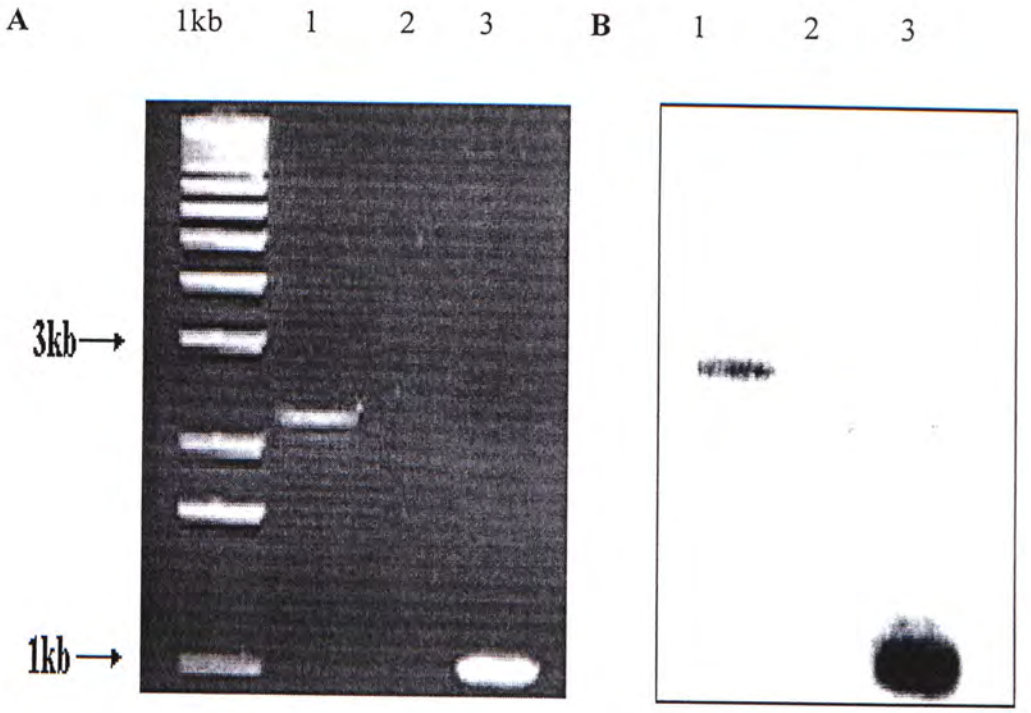


Fig.2.4. PCR amplification of gfPRL gene from goldfish genomic DNA using gene specific primers designed from the cDNA and the 5' flanking region. Lane 1: gfPRLF3 is the forward primer from the 5'end of the cloned gfPRL gene promoter and gfPRLGCR is the reverse primer from the 3'end of the gfPRL cDNA clone (P1A). Lane 3: The gfPRL cDNA was used as a positive control which was generated by a pair of gene specific primers, gfPRLFM (forward primer) and gfGCR (reverse primer). Lane 2 is the negative control. B. Southern hybridisation of the PCR products using ^{32}P labeled gfPRL cDNA P1A as a probe. The fragment (>3kb) was hybridized with the probe as strong as the positive control.

-560 gtacaatagtgtttctacaatatatttagttaaattacgtgtatgtatacactatgcctgtacctttttt
-490 tatgcacttgaagctcctgtcaccaagacaagttccttatgtgtgtgtgggggggggggggtgtaaac
-420 gctctttttgacactgctggtggtgtactattagaatcagttcatttttccattcagacaccctaaaata
-350 attgcatcgcaaattatttttttaaataaaaaaattttagaagaaaaaaaattggcacacagctcattaa
-280 atgtcacaaataatcatcactacatttaaaagttaaatagaagaaagaatgttttacatatatttaactg
-210 gtacatcatctgacctctgatcatttgatttttgtgtcctgtatctactcagacagcttataaatattga
-140 tcagccaaacactcacatcaatgtttatcattagtttcaggatttaactaatacacctggagtgcaagac
-70 attgcatatgcaaatgagaggaccgagaagagcaggcctatataatgggaagaaaatgacagagagctca

Met Thr Gln Gly

+1 AGAGAGGAAATCAGACTGCAAAGATTCAACCAAGACCTGTTAAAACCAGTTAAA ***ATG ACT CAA GGA***

Ser Arg Leu Tyr Phe Ala

+67 ***TCT AGA CTG TAC TTT GCA G*** gtaagcaaagttccctgcaacattttacactttacttctcctcc
+130 ctcgttctttacatcctcttttccattttatgttcatgctaactggacttcagatgaccctaatgttatt

Val Ala Val Leu Met Cys Gly Phe Val Ser

+200 tttgaagggtttctttttctttttacatttcag ***TG GCT GTT CTG ATG TGT GGG TTT GTC TCC***

Ile Asn Gly Val Gly Leu Asn Asp Leu Leu Glu Arg Ala Ser Gln Leu Ser Asp

+262 ***ATC AAC GGT GTC GGT CTG AAT GAT TTA CTG GAG CGA GCC TCT CAA CTC TCA GAC***

Lys Leu His Ser Leu Ser The Ser Leu Thr Asn Asp Leu

+316 ***AAA CTT CAC TCT CTC AGC ACC TCT CTC ACT AAT GAC CTG*** gtcagtcccaaacaccctg
+374 gtcgtatgcccactcactgggttatctgcaatatttgacttcagttttatccaaacaggtatttctttca

Asp Ser His Phe Pro Pro

+444 ttgatttgatgttgcttgtttaatattaacgctgttttcctctacag ***GAT TCT CAC TTT CCT CCG***

Val Gly Arg Val Met Met Pro Arg Pro Ser Met Cys His The Ser Ser Leu Gln

+509 ***GTT GGA AGG GTA ATG ATG CCC CGT CCG TCG ATG TGC CAC ACA TCC TCT CTT CAG***

Ile Pro Asn Asp Lys Asp Gln Ala Leu Lys Val Pro

+563 ***ATT CCC AAT GAC AAA GAC CAA GCC CTG AAA GTC CCG*** gtaagagcatgctttactgcatc
+621 catcctcatctcaaatcatcatatcaaatgtcataaactttctcctgcactctcagaaaaaaggtatgac
+691 aactgtaactggggtgatacctttttcaaaaggtacatgtttctttatctctacacttttgtatctctgta
+761 atatgtaactttaggtttgaatgtgtaccttgaaaaggtatcacccctgtaacaacttccgtacttttat
+831 ttctgagagtgtgttccagcttattttattattattttgttatttgaacaattgtataataaaaaaaaaa
+901 tatatatatgcatacatttattgataatttaaatacaaatataatttttatagtatacaaatatattatt
+971 aaattgcttaactgtcactatccaaacttaaattactgtaatttatgaacactttggaatataaacctat
+1041 cgttggtctctttttaaagacataaattagcaaaagtggaatttaaaaaaaaaattaaagtatcaaaaag
+1111 ttatagaagtttaaatgtactgtaaataaaatgtgctatattaatttaattttatttaatttattataaa
+1181 tattttacataacaggttttactctaaatttggtataaaattaaagccttgtgtataaataggttatgtt
+1251 acaaatttgaagttaatatgaaaaaatctgaggttcctgtgagattttatttagggcgtcataccacaca
+1321 cagccactggacgtcatcaaaactattttgaattttgcttaatgaatttttctctagattcttctgtgaa
+1391 tttaacttctatctcaaaataacatcctgagactcagacctttccaatcatatgtttgttgccaagatta
+1461 taaaaagttttggttctagaaaaatgcattatgttttatgacacttggccctgcccactaagagggaggt
+1531 gaccgccattagaatttaaagtaccatttccatggtgaagcaatgtccgatttcaaaattcacaggatga
+1601 atcttgggcttctaagctttcaaatgatatatgatttatgaggattactaaaagattttatagagaaaaa
+1671 gacaacaacaaaaaaaaaatagtgccaagcgtcccacaggtgggacggtgacagttatgggactttgata
+1741 ctttatataaaacaaaatatacaaaaaacataattcataaaaatgtgtttaataaaaaaaatgatgtgtt
+1811 taattatagaagaaaaataaatctatatgaatcaagctgttttatctgttaaagtcctctgaagagacca
+1881 tcagaccatgttgaatagattaatttaggtttttattcacatatgggcaataatcaaaatacataaatag
+1951 agtacattatatgtgatacatgtgaactcaatagcttctctgactcactggttcttgcagaattacgaac
+2021 atgcattcttgaaaccccattggttcaacagtcttatggacttggaacaaatgaatttacttgattcttt
+2091 tatttttgggtgaactaacactttaatgtgcagtgttaactatgactaaacatctcctacttgtggtca

Glu Asp

+2161 caatcgtagttacaggcaaaacagacaagcggtgtatgaagttttatctgattgtgtgtgcag ***GAG GAT***

Glu Leu Leu Ser Leu Ala Arg Ser Leu Leu Leu Ala Trp Ser Asp Pro Leu Ala

+2230 ***GAG CTG CTG TCT CTG GCT CGG TCT CTG CTT CTG GCG TGG TCC GAT CCC CTC GCC***

Leu Leu Ser Ser Glu Ala Ser Ser Leu Ala His Pro Glu Arg Asn Thr Ile Asp

+2284 ***CTT CTC TCC TCT GAG GCG TCC AGC CTG GCA CAT CCA GAA CGC AAC ACC ATT GAC***

Ser Lys Thr Lys Glu Leu Gln Asp Asn Ile Asn Ser Leu Gly Ala Gly Leu Glu

+2338 ***AGC AAG ACC AAA GAG CTG CAG GAC AAC ATC AAC AGC CTG GGA GCA GGT CTG GAG***

```
      His Val Phe Asn Lys
+2392 CAC GTC TTT AAC AAG gtgattgtacttgagagaacatctcacagattgtatattgtgaatggtgac
+2458 tgtaggagggaagtacaactgaatggtgtttggataaaaaccttgaaggaaatctttttaaacagggttg
                                                  Met Asp Ser Thr Ser Asp Asn Leu
+2528 ggttattcattgtccctgaccatgttttcttctgcgacag ATG GAC TCA ACT TCA GAC AAC CTT
      Ser Ser Leu Pro Phe Asp Ile Asn Ser Leu Gly Gln Asp Lys Thr Ser Arg Leu
+2592 TCC TCT CTC CCT TTT GAC ATC AAC AGC CTC GGC CAG GAT AAA ACC TCC CGA CTT
      Val Asn Phe His Phe Leu Leu Ser Cys Phe Arg Arg Asp Ser His Lys Ile Asp
+2646 GTC AAT TTC CAT TTC CTG CTG TCC TGT TTC CGC AGG GAC TCT CAC AAA ATT GAC
      Ser Phe Leu Lys Val Leu Arg Cys Arg Ala Ala Lys Lys Arg Pro Glu Met Cys
+2700 AGT TTC CTC AAA GTT CTC CGC TGC CGG GCA GCC AAG AAG AGA CCC GAG ATG TGT
        *
+2754 TAG agaggaaatgctgtgctctgcttctctcagtgt
```

Fig. 2.5. Structure and nucleotide sequence of gfPRL gene. Exons are in uppercase letters; introns and flanking regions are in lowercase letters. The numbering shown in the left margin refers to the last nucleotide on the corresponding line. Encoded aa residues, represented by three-letter code, are placed above each codon.

2.3.3 Identification of the transcription initiation site

The 5'-terminus of the mature PRL mRNA was mapped using the primer extension method (Xiong *et al.*, 1992). A 22-mer unique and specific primer PRLR3 (Table 2.1) labeled with ^{32}P at the 5' terminus and hybridized to goldfish pituitary mRNA. After extension by Superscript reverse transcriptase from labeled primer and electrophoresis on a thin sequencing gel, a single discrete band was found (Fig. 2.6). The size of the fragment was determined by comparison to the sequence of the clone containing the first exon and 5' -flanking region of goldfish PRL gene initiated by the same primer. The result suggested that the 5'-terminus of PRL mRNA is located at a position 55 bp upstream from the start codon.

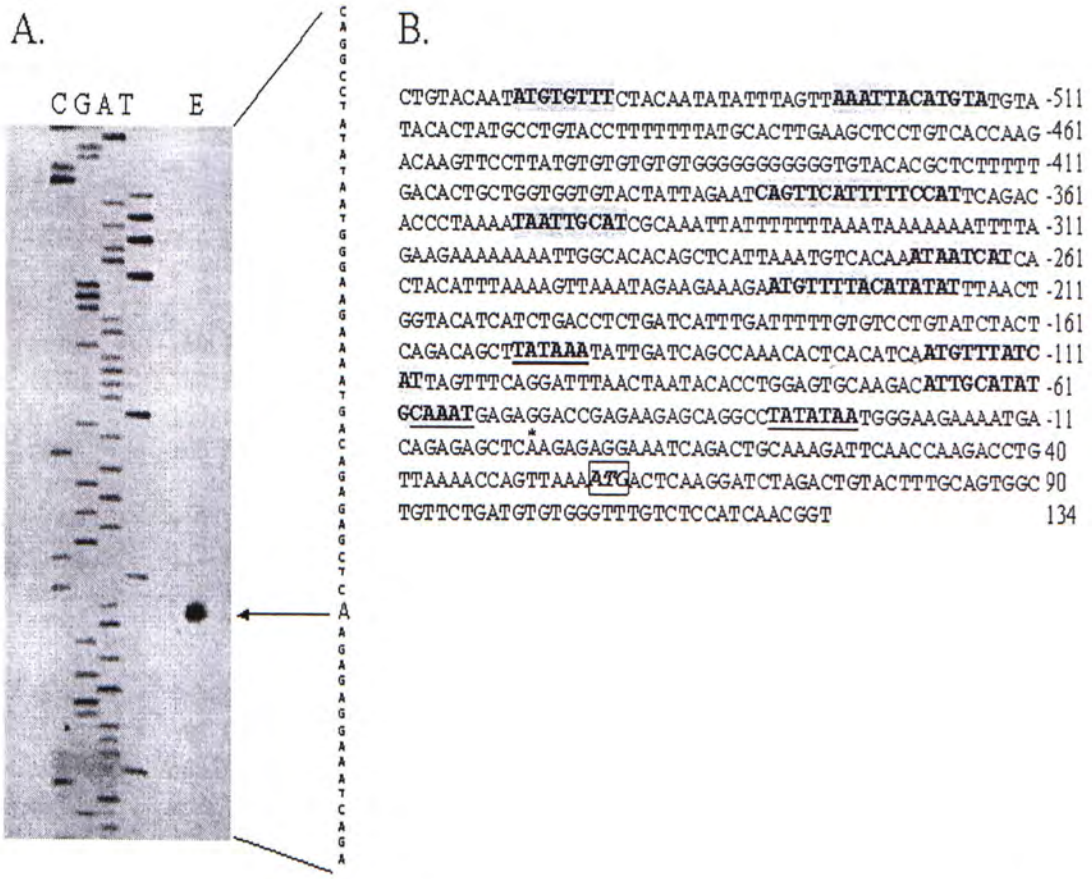


Fig.2.6. Mapping of the transcriptional start site by Primer Extension.

Panel A. Autoradiogram of the DNA sequencing gel showing the DNA sequence and the primer extension products primed with the same primer. Lane C, G, A and T are the size maker from dideoxy sequencing reaction products of clone Pr5, which contained the 5' flanking sequence of P1A, as template with PRLR3 primer. The corresponding sequence was shown on the right side of the autoradiogram. Lane E is the primer extension product generated using the same primer PRLR3. The arrow indicates the adenine residue at the transcription start site.

Panel B. The sequence of the 5' flanking region of gfPRL gene with the transcription start site indicated with an asterisk. The right hand side shows the coordinate number of the last residue. The first TATA box was located between coordinate –31 and –25.

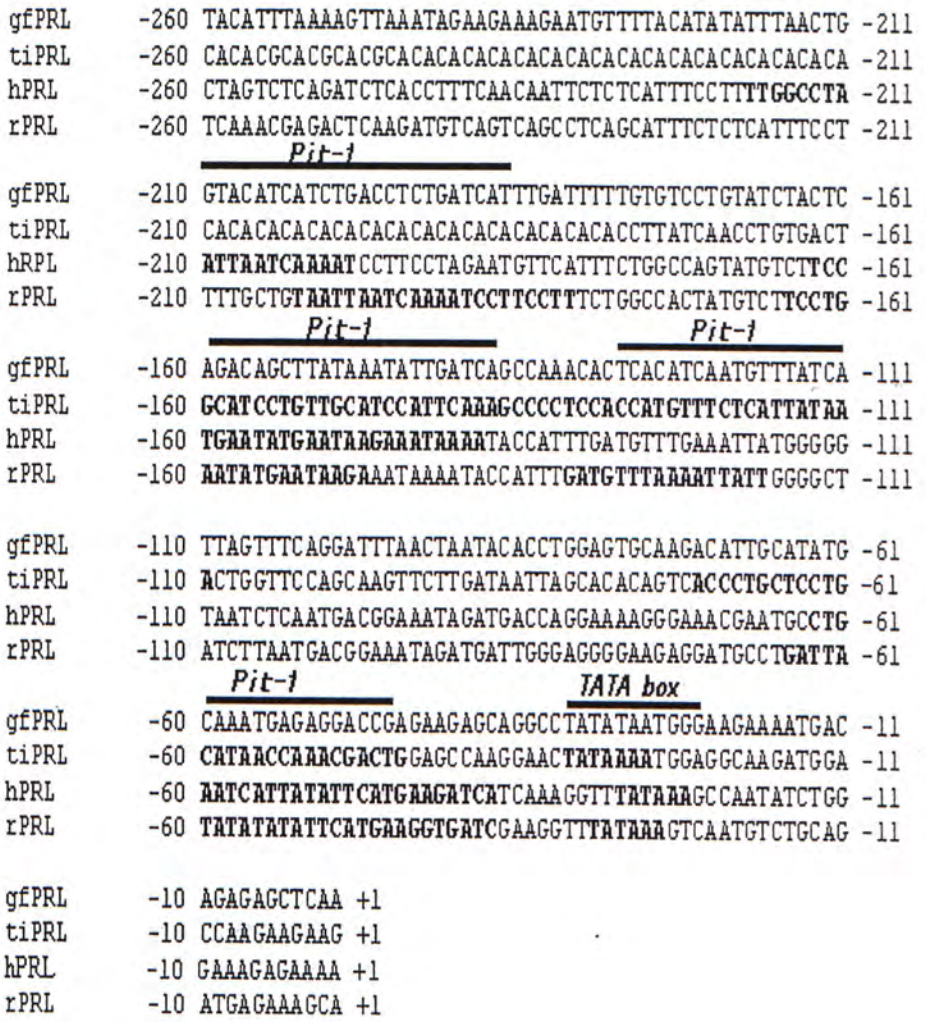


Fig.2.7. Multiple sequence alignment of the 5' flanking regions of PRL genes from different species (gfPRL, goldfish PRL; tiPRL, tilapia PRL; hPRL, human PRL; rat PRL, rat PRL). The letters in bold-face are the Pit-1 binding sites identified by DNase footprinting (Poncelet A.C. *et al.*, 1996; Lemaigre *et al.*, 1989; Nelson *et al.*, 1988) and the TATA box of which the location is highly conserved. The positions showing highly conserved Pit-1 regions were highlighted with a black line above the sequence.

	Exon/intron junctions				Intron size (bp)	Intron class
TTGCAG	GTAAGC	1st intron	TTTCAG	TGGCTG	47	1
GACCTG	GTCAGT	2nd intron	CTACAG	GATTCT	138	0
GTCCCG	GTAAGA	3rd intron	GTGCAG	GAGGAT	1626	0
AACAAG	GTGATT	4th intron	CGACAG	ATGGAC	161	0
goldfish consensus G	GTRAG		YTRCAG			
rat consensus AG	GTRAG		YNYYYNCAG			
Common consensus G	GTRAG		Y..CAG			

Fig. 2.8. Comparison of the exon-intron boundaries of the rat PRL gene.
The nucleotide sequences of the exon-intron boundaries are from Fig. 2.5. The sequences were aligned using GT....AT common sequence and the vertical lines indicate splice sites which would be utilized. The consensus sequence and intron classifications are as described by Sharp (1980). Class 0 introns interrupt the reading frame between codons and class one introns interrupt between the first and second nucleotide of a codon. The abbreviations used are: R, purine nucleotide; Y, pyrimidine nucleotide. The possible alternative splicing was proposed in which a glutamine was removed from the beginning of exon four.

2.4 Discussion

2.4.1 Sequence analysis of the gfPRL gene

The objective of the DNA sequencing endeavor was to determine the sequence of the gfPRL gene and to identify all of its exon-intron boundaries and the region preceding the transcription initiation site. The sequence of about 3200 nucleotides was obtained (Fig. 2.5), the gene consists of five exons separated by four introns. Introns range in size from 47 bp to 1.6 kbp nucletotides. Comparison of the genomic DNA sequence with the PRL cDNA sequence reveals five exon regions of 85, 122, 108, 183 and 189 nucleotides (Exons I, II, III, IV and V, respectively). These exons code for aa residues –23 to –14, residues –13 to 28, residues 29 to 64, residues 65 to 124, and residues 125 to 188 where negative numbers refer to positions in the NH_2-terminal precursor (signal peptide) segment. These five exons together contain the entire coding sequence of gfPRL.

However, the genomic and cDNA sequences (P1A and P8A contain the same sequence except for 4 silent mutations) were identical except for one nucleotide substitution, thymine (T) to guanine (G) at a position +2591 which is in the coding region causing a silent mutation from CTG to CTT encoding for a leucine residue. Two P1A silent mutations were found at positions +2259 and +2708 while there are two P8A silent mutations at positions +2660 and +2744. As a result, the obtained gfPRL gene sequence is distinct from P1A and P8A, and would be an allelic homolog to P1A and P8A.

The organization of the gfPRL gene into five exons is similar to other cloned PRL genes. The most widely accepted model for the evolution of the GH/PRL gene family is based on an observed internal aa sequence homology found in human GH, human PL, and ovine PRL (Niall *et al.* 1971). In human GH, for example, a homologous aa sequence was found in four regions: one in exon II, one in exon IV and two in exon V. Besides, aa

sequence homology was found among human GH, human PL, and ovine PRL. As a result, Niall *et al.* (1971) suggested that two successive tandem duplications of a primordial peptide might have resulted in a primitive polypeptide, and subsequent duplication and divergence of this polypepetide resulted in the family observed. The other two exons (exons I and III) of the human gene are thought to have separate origins. Exon I, which eventually took over the controlling function of the gene, was incorporated at the 5' upstream region, and exon III, which conferred the hormone with carbohydrate-regulating properties, was incorporated between the originally duplicated regions. Imperfect direct repeats have also been found flanking exons I, III, and V of rat GH, rat PRL, human GH, and human PL genes. This observation was taken as further evidence that these regions arose by separate insertion events by a mechanism analogous to DNA transposition. The formation of the precursor gene marks the end of phase I. At the beginning of phase II, the entire precursor structure was duplicated and subsequent divergence gave rise to the genes that encode GH, PRL and PL.

The investigation of gfPRL gene on flanking region of exons I, III and V do not show any direct repeats similar to those of the rat GH, rat PRL, human GH, and human PL genes. Moreover, according to the description by Niall *et al.* (1971), the internally homologous regions do not exist in the gfPRL gene. There although the similarity between the polypeptide sequences of fish GH and fish PRL (Kawauchi *et al.* 1986; Yasuda *et al.* 1986) is consistent with phase II of the model (Fig. 2.8.), the fish PRL gene structure is inconsistent with phase I. This observation concurs with Agellon *et al.* (1988), Takayama *et al.* (1991) and Xiong *et al.* (1992). These investigators failed to observe any significant degree of the internal similarity as described above.

2.4.2 Analysis of the exon-intron boundaries

A comparison of the exon-intron boundaries reveals that all of the boundaries follow the GT..AG rule proposed by Breathnach *et al.*, 1976. The actual sequences in the RNA are of course GU-AG and were found only immediately within the intron at the presumed junctions. Also, all of the exon-intron boundaries show considerable homology with a consensus splice junction (Fig. 2.8.). It was suggested that a common splice sequence hybridize to small nuclear RNAs and that the resulting hybrid is important for splicing (Kuhn, 2001).

2.4.3 Analysis of the 5' flanking region of gfPRL gene

The 5' flanking region of gfPRL gene is composed of around 600 bp nucleotides. According to the study of the transcriptional regulation of the PRL gene (Nelson *et al.*, 1986; Poncelet *et al,*. 1996), the first 250 bp from the start codon are sufficient for pituitary-specific expression of PRL gene. As a result, the sequences of the 5' flanking region of gfPRL gene is sufficient for us to study the regulation of the proximal promoter region of gfPRL gene. *Pit*-1 is a major transcription factor that regulates the transcription activity of PRL gene. We identified eight putative *Pit*-1 binding sites (Fig. 2.2.) referring to the consensus DNA sequence A/TTATT/CCAT proposed by Nelson *et al.* (1986) which is highly conserved in teleost (Argenton *et al.*, 1996; Poncelet *et al.*, 1996). However, the number of Pit-1 binding sites identified by DNase footprinting so far ranged from two to four and within the coordinate –300 bp region. The first two Pit-1 binding site locations are well conserved (Fig. 2.7.). There are two putative TATA boxes separated by ~100bp (Fig. 2.3.). The TATA box is similar to the Pribonow box of the prokaryotic promoter and is involved in initiation of gene transcription (Kuhn 2001).

2.4.4 Identification of the transcription initiation site

The transcription initiation site was identified by the method of primer extension. The squence alignment analysis of flanking DNA sequences (Fig. 2.7.) suggests that the site located by primer extension is in fact the transcription initiation site. There is only one initiation start site located at position 55 nucleotides upstream from the start codon.

Analysis of the 5' flanking sequences upstream from transcription initiation site revealed that the sequence TATATAA was found at positions –31 to –25 from the transcription start site (Fig. 2.6B). This sequence is similar to the TATA box that has been found about 30 nucleotides upstream from the transcription initiation site of a number of eukaryotic genes transcribed by RNA polymerase II (Kuhn, 2001).

2.5 Conclusion

Using the genome walking PCRs and primers designed from the gfPRL cDNAs, we have successfully isolated a 5' flanking region for gfPRL gene. The 5' flanking region is linked to a coding region encoding the gfPRL as proven from further PCR with primers designed from the 3' UTR of the gfPRL cDNA and the 5' flanking region to obtain a 2574 bp fragment of gfPRL gene with its proximal gene promoter region and five exons of the coding region.

Chapter Three

Promoter Analysis of the gfPRL Gene

3.1 Introduction

PRL gene is expressed in a tissue-specific manner and is under complex hormonal control. However, molecular mechanism of other hormonal control of PRL gene transcription and the tissue specific expression of PRL in the lactotroph is not fully understood. Pit-1 alone still cannot revolve the mystery that PRL is expressed in the lactotrophs but not in other cell types such as somatotroph and thyrotroph in which Pit-1 also exists. Even though the discovery of different Pit-1 subtypes and their functional analysis showed some hints to the answer, there is still a long way to understand the whole picture.

PRL has many different functions, and thus its expression is also under complex hormonal controls including thyroid hormone, glucocorticoid, DA, etc (Maurer *et al,*. 1981; Maurer, 1981) which regulate the levels of PRL mRNA. Interestingly, these hormones also regulate GH mRNA, but in an opposite manner. This provides a very interesting system to study the mechanisms of hormonal regulation of gene expression.

In our study, we focus on DA and TRH. The effects of these hormones and their action mechanisms have been extensively investigated in mammalian PRL. However, in teleost, DA and TRH are believed to be the major negative and positive regulator of PRL release respectively, but only little is known about their regulatory mechanisms. We would thus like to study their role in gfPRL gene expression.

3.2 Materials and Methods

3.2.1 Preparation of luciferase reporter constructs

Gene specific primers designed (Table 2.2) from the 5' flanking region of gfPRL gene were used to amplify the desired DNA fragments. The 5' end of the forward primer contains *Kpn* I restriction site while the 5' end of the reverse primer contains *Hind* III restriction site. These two sites are the cloning sites of the luciferase reporter gene construct, pGL3 basic, purchased from Promega.

3.2.2 Preparation of frozen stock of culture cells

For each 75mm^2 flask, the cells were grown until 80% confluence. The cells was lifted by adding 0.5ml Trysin/EDTA (GibcoBRL) and incubated at 37°C for 3 min. Two microliter of culture medium were added to inactivate the trysin. The cells were collected by centrifugation (Eppendorf C5804) at 1000 rpm for 3 min. The cell pellet was resuspended in 2ml of frozen medium (10% DMSO, 50% fetal calf serum (FCS), 40% culture medium). One ml aliquots of the mixture were prepared in 2 ml-vials and kept at -20°C for 2 h. Then it was transferred to –80°C for another 2 h and finally stored in liquid nitrogen.

3.2.3. Cell Culture

Cell lines from ATCC were cultured in 75mm^2 flask with appropriate medium (F12K for GH3; F12 for GH4ZR7 and HeLa; MEM for CHO-K1) in a 0.5% CO_2 incubator at 37 ℃. All media are purchased from GibcoBRL. When cells grown to certain confluency (80% for GH3, HeLa, and CHO-K1; 60% for GH4ZR7), they are subcultured using the following procedure.

The cells were washed by ~10ml phosphate buffered saline (PBS). Then 0.5 ml (for a 75mm^2 flask) Trysin/EDTA was added into the flask and incubated at 37°C for 5 min Two

ml of culture medium were added to stop the trysin reaction. Then the cells were transferred into a 15 ml conical culture tube. The cells were collected by centrifugation at 1000 rpm for 3 min (Eppendorf C5804) at room temperature. The pellet was resuspended in 5 ml of culture medium. Appropriate amount of the resuspended cells were added into culture flask with 10ml culture medium.

3.2.4 Transfection of mammalian cells for transient gene expression study

Transfection of mammalian cells was performed using the LipofectAMINE reagent (GibcoBRL). Around 1 x 10^5 of mammalian cells seeded into each well of 24-wells plate, were grown in 0.5 ml growth medium until 80% confluence. Solution A (300 ng luciferase construct and 20 ng pRL-CMV in 100 µl of MEM) was mixed with solution B (1 µl LipofectAMINE reagent in 100 µl of MEM) for each well, and incubated at room temperature for 15 min before adding to each well. The cells were incubated at 37°C with 5% CO_2 for 5 h. Then the transfection solution was replaced with the growth medium or drug (DA/TRH) containing growth medium. For cell-type specificity investigation, the transfected GH3 cells were incubated with F12K (GibcoBRL) growth medium and incubated for 16 h before preforming luciferase assay. For DA investigation, the transfected GH4ZR7 cells were incubated with different concentrations of DA in F12 (GibcoBRL) growth medium for 16h before perform the luciferase assay. For TRH investigation, the transfected GH3 cells were incubated with different concentrations of TRH in F12K (GibcoBRL) growth medium for 16h before perform the luciferase assay.

3.2.5 Luciferase assay

The Dual-Luciferase Assay System (Promega) was used and the assay procedures followed the instructions from the supplier. The reaction was carried out in 12 x 75 mm tube, and the light emission was detected on a Lumat LB 9501 luminometer.

3.3 Results

3.3.1 Tissue-specific transcription of gfPRL promoter

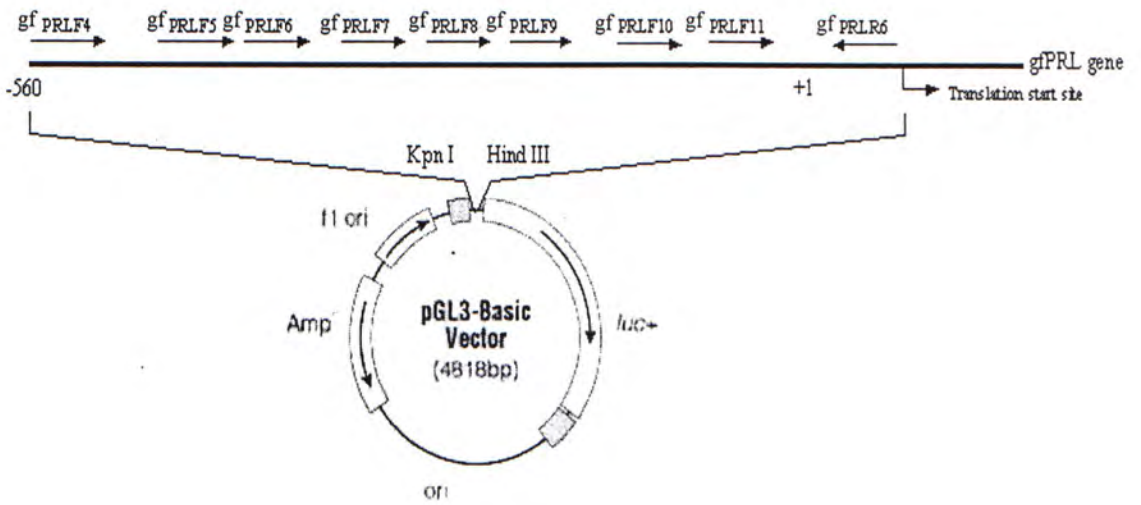
To investigate the tissue-specific regulation of gfPRL promoter, a construct was made by linking the 0.6 kb fragment to the luciferase reporter vector (pGL3-basic) (Table 3.1). The pGL3 vector provides a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression. These factors may be *cis*-acting, such as promoters and enhancers, or *trans*-acting, such as various DNA-binding factors. The pGL3 Vectors carry the coding region for firefly (*Photinus pyralis*) luciferase, the transcription of this reporter gene is driven by the gfPRL promoter and is used to monitor transcriptional activity in transfected eukaryotic cells. The assay of this genetic reporter is rapid, sensitive and quantitative. The pGL3-Basic is a promoterless vector and, however, can show basal transcription activity which as a result act as a negative control itself while the positive control is done by pGL3-CMV, which is basically pGL3-basic plus the promoter/enhancer of the immediate early region of the human cytomegalovirus (CMV) and as a result exhibits strong transcriptional activity even under no stimulation.

This construct, pgfPRL-560-Luc, was then transfected with the lipofectamine reagent into different cell lines: GH3, rat pituitary cell; HeLa, human cervical cell; HEK293, human kidney cell; LL24, human lung fibroblast; HepG2, human liver cell. The transfection efficiency of different cell lines was evaluated by co-transfecting another reporter construct, pRLCMV, which contains the Ranilase gene driven by the promoter/enhancer of the immediate early region of the human cytomegalovirus (CMV). The 0.6 kbp gfPRL gene promoter region is able to drive high luciferase gene expression in GH3 cells (~45-fold) but not in other cells (Fig.3.1), suggesting that gfPRL promoter activation is pituitary specific.

Table 3.1 Nucleotide sequences of oligonucleotide primers used in promoter deletion study

Primer name	Nucleotide sequence	Starting coordinate	Constructs
gfPRLF3	5' GCGGTACCGTACAATAGTGTTTC	-560	pgfPRL-560-Luc
gfPRLF5	5' GCGGTACCTGCTGGTGGTGTAC 3	-405	pgfPRL-405-Luc
gfPRLF6	5' GCGGTACCAGACACCCTAAAA 3'	-362	pgfPRL-362-Luc
gfPRLF7	5' GCGGTACCAGCTCATTAAATGTC	-297	pgfPRL-297-Luc
gfPRLF8	5' GCGGTACCGTTAAATAGAAG 3'	-248	pgfPRL-248-Luc
gfPRLF9	5' GCGGTACCATTTGATTTTTGTG 3'	-188	pgfPRL-188-Luc
gfPRLF10	5' GCGGTACCAGGATTTAACTAATA	-105	pgfPRL-105-Luc
gfPRLF11	5' GCGGTACCGAGAGGACCGAGAA(	-54	pgfPRL-54-Luc
gfPRLR6	5' GCAAGCTTAACTGGTTTTAACAG	51	

Note: The bolded letters show the restiction site.
GGTACC-Kpn I; **AAGCTT**-Hind III.



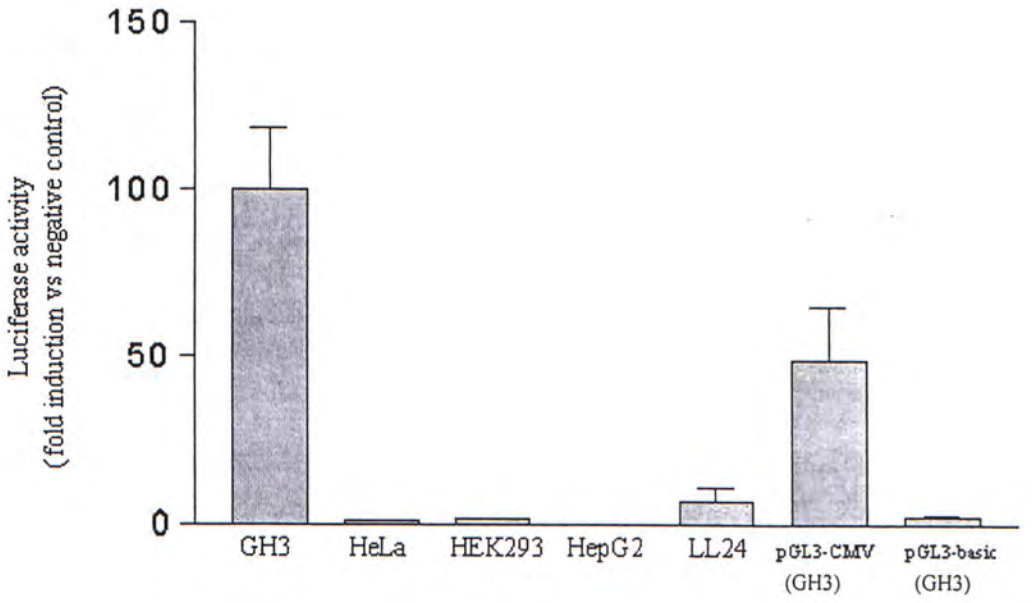


Fig.3.1. Relative activities of the gfPRL gene promoter in different cell types.
The construct pgfPRL-560-Luc (300ng) containing the -560 gfPRL promoter was transfected into different cell lines to measure the gfPRL relative promoter activties. Cells were harvasted after 16 h incubation and the luciferase activity measured. The vector pGL3-CMV and pGL3-basic were transfected into GH3 cells as positive and negative controls respectively. Fold induction of individual luciferase activity was compared with the negative control. GH3, rat pituitary cell; HeLa, human cervial cancer cell; HEK293, human embyonic kidney cell; HepG2, human hepatoma cell; LL24, human lung fibroblast. Data are from four experiments and verticle bars represent SEM.

3.3.2 Identification of regulatory regions of gfPRL gene promoter

To investigate the regulatory regions in the 5' flanking sequence of the gfPRL gene, nine deletion mutants were generated in the gfPRL 5' flanking region, as shown in Table 3.1. As controls, we used the promoterless construct (pGL3-basic-Luc) and the positive control, pGL3-CMV-Luc, which carry the promoter/enhancer of the immediate early region of the human cytomegalovirus.

Transfection of GH3 cells with the 5'-deletion mutants revealed two regulatory regions in the 5' flanking sequence of the gfPRL gene (Fig.3.2). The region upstream from the TATA box between coordinates –188 (pgfPRL –188-Luc) and –405 (pgfPRL –405-Luc) has an activating effect on expression of the reporter luciferase gene. An inhibitory region is present between coordinates -405 and –560 (pgfPRL –560-Luc). The pgfPRL 405-Luc construct has the highest luciferase activity. The removal of the upstream sequence from the coordinate –105 (pgfPRL 105-Luc) showed a dramatic decrease in the transcription activity, however, it is still higher than the negative control by ~4 times, while the construct pgfPRL 54-Luc has similar transcription activity as the negative control. This suggested that the region between –188 and –105, containing one putative Pit-1 binding site is sufficient to drive the promoter transcription.

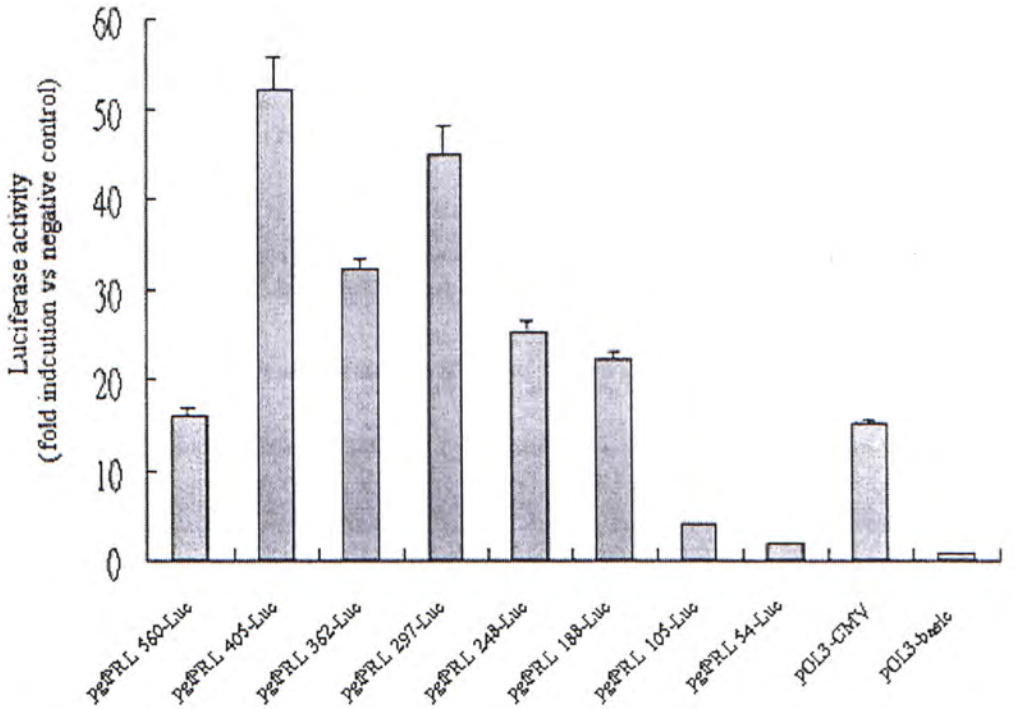


Fig.3.2. Relative activities of deletion mutants from the gfPRL gene promoter in GH3 cell-line. The constructs were prepared by fusing 5'deleted gfPRL promoter with the luciferase reporter gene. The number of the construct represents the coordinate of gfPRL gene. Each contruct, 300ng, was transfected into GH3 cells. The luciferase reporter gene drived by CMV promoter (pGL3-CMV) acts as a positive control while the promoterless luciferase reproter gene acts as negative control (pGL3-basic). Fold induction of individual luciferase activity was compared with negative control. Data are from four experiments and verticle bars represent SEM.

3.3.3 Inhibitory effect of DA on gfPRL promoter transcription activity

DA is considered to be the major physiological tonic inhibitor of PRL release, yet there is increasing evidence showing that it can also stimulate PRL release from lactotrophs and the cell lines (Burris *et al.*, 1992; Chang and Shin, 1999). However, there is no study concerning the regulatory effect of DA on teleost PRL gene transcription. Besides, the stimulatory action of DA on either mammalian and teleost PRL gene transcription have never been demonstrated.

The construct carrying –560 promoter region, pgfPRL-560-Luc was transfected into GH4ZR7 cells which stably expressing the D2s, DA receptor subtype. DA was added at concentrations of 1μM, 10μM and 100μM for 16 h. An induction of 12% luciferase activities was recorded at concentrations of 1μM and 10μM. At 100μM, DA significantly inhibits the gfPRL promoter activity by about 65% (Fig.3.3). It is suggested that the negative regulation by DA acts on both mammalian and teleost PRL gene transcription. Besides, DA was found to be able to slightly stimulates gfPRL promoter activities at low concentrations.

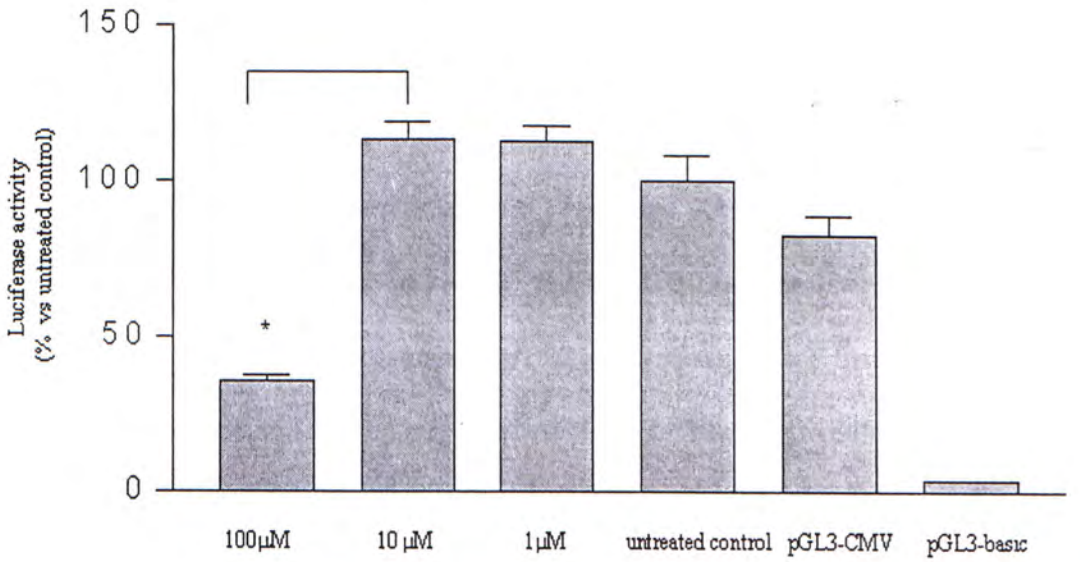


Fig.3.3. Inhibitory effect of different concentrations of DA on gfPRL promoter transcription activites. The construct pgfPRL 560-Luc (300ng) was transfected into GH4ZR7 cells. The transfected cells were incubated in 100mM, 10 mM and 1mM DA respectively. Luciferase gene regulated by CMV promoter (pGL3-CMV, 300ng)) acts as positive control while the promoterless vector (pGL3-basic, 300ng) acts as negative control. The luciferase activities were compared with the untreated control. Data are from four experiments and verticle bars represent SEM. *P<0.05 (Student's t test)

3.3.4 DA responsive sequence of gfPRL promoter

Responsive elements for a number of peptide hormones have been mapped to the promoter region of the PRL gene, suggesting that DA responsiveness may also be localized to the proximal 5' flanking sequences.

Eight deletion constructs prepared in the previous experiments were used to locate the responsive region of DA action. They were transfected in two groups of GH4ZR7 cells. One of them was treated with 100μM DA while the others were untreated in order to eliminate the effect due to different basal activities of each construct. The construct pgfPRL-560-Luc showed the highest response (60% reduction, Fig. 3.4) to the inhibitory effect of DA while the transcription activities of the constructs possessing promoter from –405 to –188 coordinates were reduced by average 40%. The inhibitory effect of DA was lowered to 21% on the construct pgfPRL-105-Luc transcription activities and subsequently eliminated on the construct pgfPRL-54-Luc, suggesting that the DA responsive region is between –188 and –54 bp region where there are two putative Pit-1 binding sites.

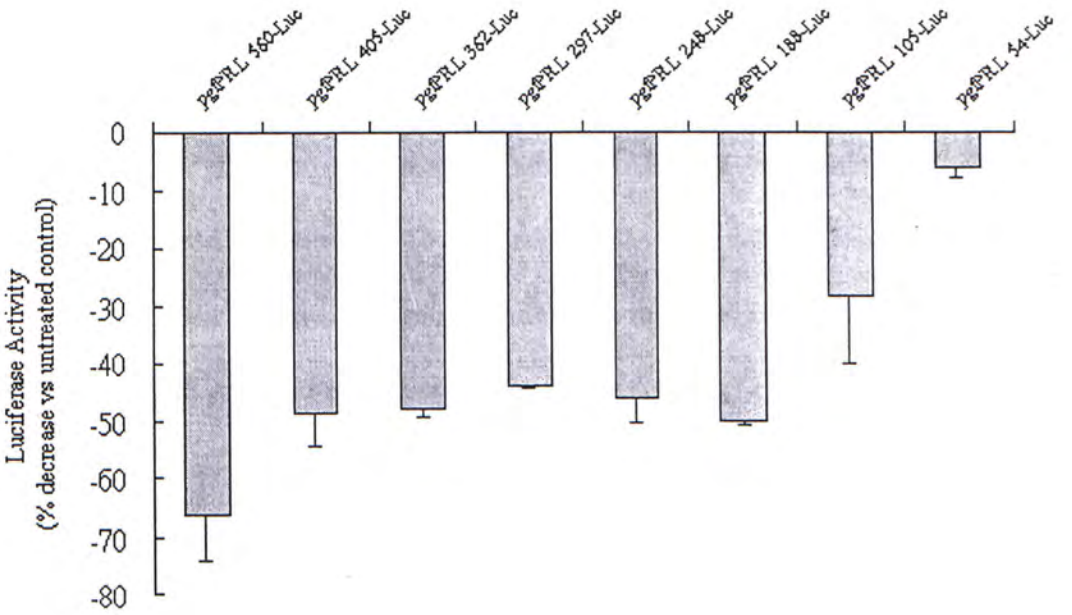


Fig.3.4. The effect of DA on different regions of gfPRL promoter. Different constructs (300ng) prepared in previous experiment were transfected in two groups of GH4ZR7 cells. One group of the cells was incubated in 100mM DA for 16 h while the other group was incubated in medium only. They were harvasted for measurement and comparison of the luciferase activties. Data are from four experiments and verticle bars represent SEM.

3.3.5 The action of TRH on gfPRL promoter

TRH was originally isolated as a hypophysiotrophic factor that stimulates thyroid stimulating hormone secretion from pituitary cells (Schally *et al.*, 1966). Subsequently, TRH has been shown to stimulate PRL release from lactotrophs in a dose-dependent manner both *in vitro* and *in vivo* (Blake, 1974; Bowers *et al.*, 1971; Tashjian, Jr. *et al.*, 1971). TRH is considered as a PRL-releasing factor (PRF) and can efficiently stimulate mammalian PRL promoter in cell lines (Berwaer *et al.*, 1993; Yan *et al.*, 1991).

The construct of pgfPRL 560-Luc were transfected into GH3 cells. Nine different concentrations of TRH ranging from 1000nM to 2.56pM (5-fold serial dilution) were added to the medium and incubated for 16 h. The luciferase activities at different concentration of TRH were more or less the same as the untreated control (Fig.3.5), suggesting that TRH has no effect on gfPRL promoter transcription activity.

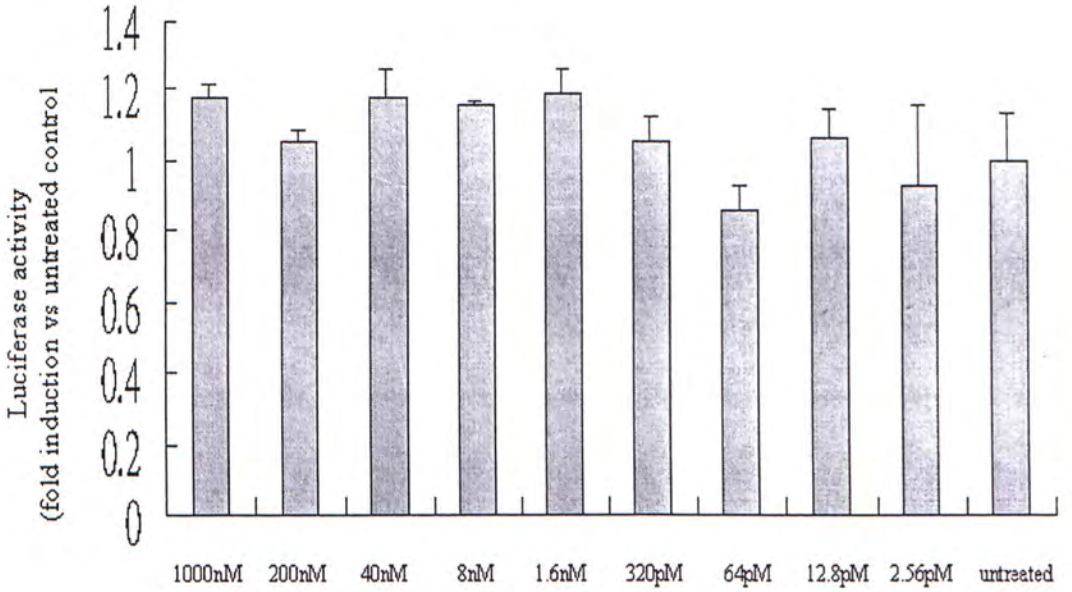


Fig.3.5. The effect of TRH on gfPRL promoter transcription activities. GH3 cells were transfected with pgfPRL 560-Luc construct and incubated with different concentration of TRH for 16 h. The luciferase activities were compared with the untreated control. Data are from four experiments and verticle bars indicate SEM.

3.3.6 TRH responsive sequence of gfPRL promoter

Eight deletion constructs prepared in the previous experiment were transfected into two groups of GH3 cells. One of the groups was incubated with 100 nM of TRH for 16 h while the other was incubated in the medium only. This is to eliminate the effect of different basal activities of each construct. Our result showed that TRH has no effect on the construct pgfPRL-560-Luc (Fig. 3.6). However, the transcription activity of constructs pgfPRL-405-Luc and pgfPRL-297-Luc were inhibited by TRH has 18% and 14% respectively. The strongest inhibitory response (36% reduction) came from pgfPRL-362-Luc. In general, gfPRL promotor was negatively regulated by TRH and there were only three constructs showing such response (pgfPRL-405-Luc, pgfPRL-362-Luc and pgfPRL-297-Luc).

TRH regulates both mammalian and teleost PRL secretion (Kagabu *et al.*, 1998; Tashjian, Jr. *et al.*, 1971; Williams and Wigham, 1994). However, our results suggested that TRH does not induce the transcription of gfPRL promoter. It will be interesting to investigate whether this effect is due to the absence of responsive sequences in the proximal gene promoter region near the TATA box. In other words, the search of the TRH responsive site in further upstream region of the gfPRL will enable us to understand how TRH regulates gfPRL gene transcription. *In vivo* study of the effects of TRH on gfPRL is also needed.

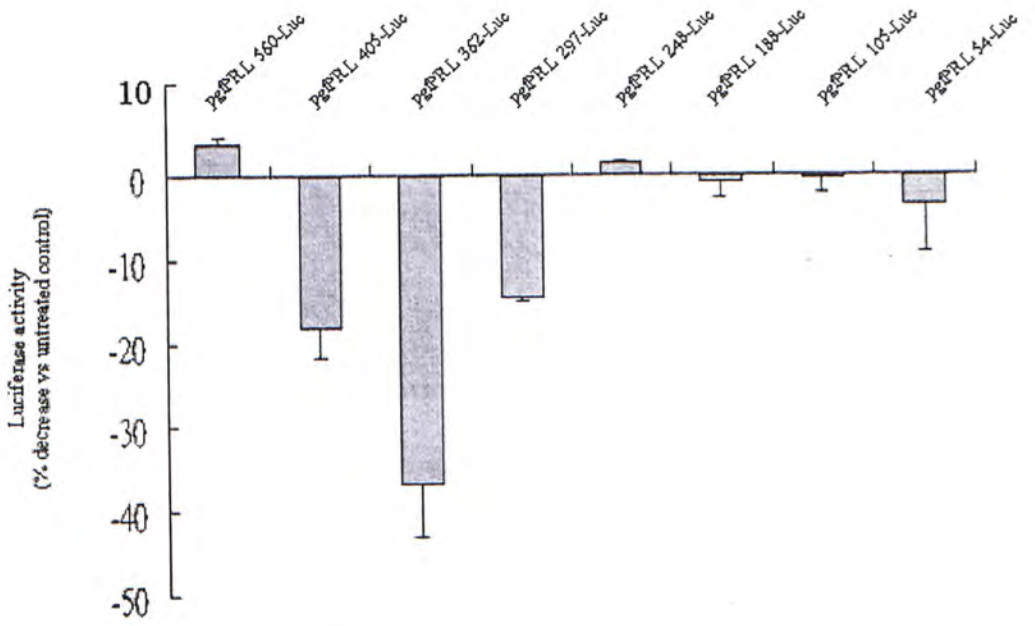


Fig.3.6. The effects of TRH on different region of gfPRL promoter. Different constructs (300ng) prepared in the previous experiments were transfected in two groups of GH3 cells. One group of the cells was incubated in 100 nM TRH for 16 h while the other group was incubated in medium only. They were harvasted for measurement and comparison of the luciferase activties. Data are from four experiments and verticle bars represent SEM.

3.4 Discussion

3.4.1 Tissue-specific transcription of gfPRL promoter

To study the gfPRL promoter in a homologous system, we must use primary pituitary cell since there is no teleost pituitary cell line available. However, the disadvantage of using primary pituitary cell is that we need to scarifice a huge number of fish in order to get enough cells for experiments due to the tiny size of individual pituitary. In addition, there are only limited teleost cell lines from other tissues such as EPC cell line derived from carp epidermal cell (Fijan *et al.*, 1983) for comparsion. If we used teleost cell line and mammalian cell line at the same time, analysis will be complicated due to the diversity of species. Therefore, to investigate whether a common regulatory mechanism exists between mammal and teleost, we examined the transcription activity of gfPRL promoter in different mammalian cell lines. GH3, rat pituitary cells; HeLa, human cervical cell; HepG2, human liver cell; HEK293, human kidney cell; LL24, human lung fibroblast.

The construct pgfPRL 560-Luc containing the 0.6 kb promoter fragment and luciferase gene was transfected in different cell lines mentioned above. Only GH3 cells showed transcription activity among those cell lines, suggesting that gfPRL promoter activation is pituitary-specific. The mechanism with which PRL gene expression is restricted to the lactotrophs of the anterior pituitary has been one of the most investigated areas. Transient transfection study showed that the rainbow trout PRL promoter activity can be reconstituted by co-transfecting rat Pit-1 (pituitary-specific factor) expression vector (Argenton *et al.*, 1996). In rat, Pit-1 interacts with two cis-acting regions in the 5' flanking region of the PRL gene, a proximal promoter region (-422bp to +33), and a distal enhancer region (-1831bp to –1530bp). Crenshaw III *et al.* (1989) have shown that either the distal or proximal elements is sufficient for pituitary-specific expression, although synergistic interactions

between the two are necessary for high levels of PRL gene expression. A close examination of the possible Pit-1 binding sites within the 600bp gfPRL gene 5' flanking region reveals that several most likely consensus sequence for Pit-1 binding is located at positions shown in Fig.2.6. Thus, this consensus sequence may serve as an active site for Pit-1 binding. Elsholtz *et al.* (1992) showed that Pit-1 is very conserved structurally and functionally in teleosts and mammals. In rat and chum salmon Pit-1, the aa sequences of DNA-binding domain, POU, were highly conserved with an aa sequence identity of 86%. However, the trans-activating domain that causes transcriptional stimulation by interaction with transcription factors is situated upstream from the POU domain. In this region, the two sequences not only differed greatly in length but also were less conserved (54%) compared to the POU homeodomains. However, Ono *et al.* (1995) showed that rat Pit-1 can activate salmon GH promoter with less potency, suggesting that a conserved molecular mechanism involving the regulation of PRL gene expression between teleosts and mammals.

3.4.2 Identification of regulatory regions of gfPRL gene promoter

Our deletion mutant studies reveal two counter acting regulatory regions involved in this pituitary specific expression: the positive and negative regulatory regions. The first region lies in the proximal promoter, between coordinates –105 and –405. It has an activating effect on luciferase reporter gene expression. The first 105 bp are sufficient to stimulate luciferase expression. This activity probably correlates with the presence, in this proximal promoter region, of a binding site for the pituitary-specific factor Pit-1 located between coordinate –72 and –56, which is composed of one sense and one anti-sense Pit-1 binding site. Deletion of the sequences between -188 and -105 is associated with around 18-fold decrease of luciferase activity in GH3 cells, suggesting that it contains significant factors that strongly activate the transcription activity of the promoter. Analysis of the

sequence between coordinate –188 and –105 revealed that there is another putative Pit-1 binding site located between coordinate –120 and –110, which is composed of one sense and one anti-sense Pit-1 binding element. Alignment of the 5' flanking region of human, rat and tilapia PRL genes showed that the first Pit-1 binding site is well conserved (Fig. 2.7) and fits well with the first putative Pit-1 binding site of goldfish promoter, while the location of the second Pit-1 binding site is different between mammal and fish. However, the location of the second putative Pit-1 binding site overlaps with that of tilapia. The result suggests that the second putative Pit-1 binding site is involved in the pituitary cell expression of the gfPRL promoter.

The region spanning coordinates –405 and –560 has an inhibitory effect on expression of the luciferase reporter gene. The transcription activity of pgfPRL-560-Luc construct is almost the same as the construct pgfPRL-248-Luc. Comparing with the construct pgfPRL-405-Luc, pgfPRL-560-Luc possesses only 40% transcription activity.

3.4.3 DA inhibits gfPRL promoter activity

DA exerts its action through the binding and activation of specific receptors that belong to the G protein-coupled receptor superfamily. Five distinct DA receptor subtypes (D1-D5), encoded by separate genes have been identified so far (Sokoloff and Schwartz, 1995; Gingrich and Caron, 1993; Sibley and Monsma, Jr., 1992), yet it is widely believed that only the D2 receptors are responsible for the inhibitory actions of DA in the anterior pituitary (Chang and Shin, 1999; Memo *et al.*, 1986). The genomic sequence of the DA D2 receptor is alternatively spliced to generate two isoforms (a short isoform D2s, 415 aa and a long isoform D2l, 444aa) which differ by a 29 aa insert in the third cytoplasmic domain (Monsma, Jr. *et al.*, 1989; Giros *et al.*, 1989; Dal Toso *et al.*, 1989). D2s and D2l receptors are structurally very similar, and they are also pharmacologically indistinguishable. GH4ZR7

cells are derived from GH4C1 cells by transfecting D2s receptors (Albert, 1994), and the GH4C1 cells have many different receptors such as VIP receptor, but not DA receptors (Tashjian, Jr., 1979). GH4ZR7 cell is ideal to investigate the inhibiting action of one subtype of D2 receptor (D2s) on PRL release. Therefore, we used GH4ZR7 cells in this study.

DA exerts its action through DA receptors which transduce intracellular signals by coupling to heterotrimeric guanine nucleotide-binding proteins (G-proteins). Upon receptor activation, G proteins dissociate into Gα and Gβγ subunits that in turn regulate the activity of effector molecules (Raymond, 1995; Bourne, 1997; Neer, 1995). The family of Gα subunits is divided into structural and functional homologues, for example, $G\alpha_s$ proteins couple positively to adenylyl cyclase (AC) activity to increase intracellular production of cAMP; $G\alpha_{i/o}$ proteins couple negatively to AC and are inactivated by pertussis toxin (PTX); and $G\alpha_q$ proteins couple to phospholipase C β (PLC-β) subtypes to increase $[Ca^{2+}]_i$ and are insensitive to PTX. The Gβγ subunits of G proteins couple to a variety of cell-specific effectors including AC types II and IV, PLC-β2 and PLC-β3, inwardly rectifying potassium channels, and N-type calcium channels (Clapham and Neer, 1997; Birnbaumer, 1992). In addition, G protein-coupled receptors appear to utilize particular combinations of subunits to initiate specific types of responses (Gudermann *et al.*, 1996).

The DA D2s receptor couples to PTX-sensitive G protein ($G\alpha_{i/o}$) to initiate multiple signaling pathways (Albert, 1994; Civelli *et al.*, 1993). In cells of neuroendocrine origin the D2s couples to inhibitory pathways, including inhibition of AC, activation of potassium channels to hyperpolarize the cell membrane, and inhibition of L-type calcium channels (Memo *et al.*, 1992; Lledo *et al.*, 1992; Einhorn *et al.*, 1991; Castellano *et al.*, 1993), which in concert mediate inhibition of hormone secretion and gene transcription, and inhibition of

cell proliferation (Albert *et al.*, 1990; Elsholtz *et al.*, 1991; Lew and Elsholtz, 1995; Liu *et al.*, 1994; Senogles, 1994; Vallar *et al.*, 1990). In contrast, when expressed in cells of mesenchymal lineage (e.g. Ltk-fibroblast or CHO cells), the same receptor mediates stimulation of PLC activity to induce calcium mobilization, and activation of the mitogen-activated protein kinase cascade leading to enhanced gene transcription and cell proliferation (Albert, 1994; Lew and Elsholtz, 1995; Liu *et al.*, 1994). These findings suggest that the same receptor mediates different cellular responses depending on the cell-specific effectors that are expressed.

Lew *et al.* (1995) have demonstrated in GH4ZR7 cells that either a $G\alpha_o$-or $G\alpha_{i2}$-dependent pathway can be sufficient for negative regulation of rat PRL proximal promoter. Activation of D2s receptor in GH4ZR7 results in reduction of cAMP and intracellular Ca^{2+} level (Albert *et al.*, 1990; Vallar *et al.*, 1990). $G\alpha_{i2}$ subunit was identified to be involved in cAMP reduction (Lew *et al.*, 1994). Previous study on DA regulation of PRL promoter showed that prevention on DA effects on membrane potential and intracellular Ca^{2+} by high extracellular K^+ concentration completely blocked DA regulation of the PRL promoter (Elsholtz *et al.*, 1991), suggesting that two distinct signaling pathways regulating PRL gene transcription exist. Elsholtz *et al.* (1991) also have demonstrated that dopaminergic actions are Pit-1- dependent. The investigation on Pit-1 promoter showed that two cAMP response elements (CREs) are important for DA to down-regulate Pit-1 express that in turn affects PRL transcription.

In our study, pgfPRL-560-Luc was transfected into GH4ZR7 cells and subjected to DA treatment (1μM, 10μM and 100μM) for 16 h. We demonstrated that DA inhibited gfPRL promoter transcription at concentration of 100 μM, but no inhibitory effects at 10μM and 1μM. The results suggested that a threshold value was required to turn on the regulatory

machine. This inhibitory concentration is much higher than that in other study (Elsholtz *et al.*, 1991). It may result from differences in experimental conditions, such as different number of passages of cloned cells, the presence or the absence of a DA stabilizer (ascorbic acid and sodium metabisulfite), and different incubation media. Even though there are studies showing that DA can stimulate PRL secretion at low concentration in GH4ZR7 (Chang and Shin, 1999), our study suggests that DA exerted insignificant stimulatory effect on gfPRL promoter transcription at low concentrations in GH4ZR7. This means that the stimulatory effect of low concentration DA on PRL expression may act through post-transcriptional regulation.

Promoter deletion study was carried out to identify the DA responsive region. The result showed that the deletion of the region between –560 and –188 did not reduce the inhibitory effect, but it still inhibited luciferase activity by 50-60%. The removal of the sequence between –188 and –105 bp reduced DA inhibition to around 30%. Further deletion to coordinate –54 blocks the activity of DA on promoter transcription, suggesting that DNA region between –105 and -54 bp is the major contributor to DA inhibition. Sequence analysis showed that there is a Pit-1 binding site and no cyclic AMP responsive element, hence Pit-1 may be the major mediator of DA action.

3.4.4 TRH action on gfPRL promoter

The ability of TRH to stimulate PRL synthesis and secretion involves the interaction of the hormone with a G protein-coupled receptor at the plasma membrane (Gershengorn and Osman, 1996; Straub *et al.*, 1990). The TRH receptor couples to Gq to stimulate activity of phospholipase Cβ (Aragay *et al.*, 1992), leading to increased intracellular levels of diacylglycerol activates protein kinase C (PKC) while inositol 1,4,5-trisphosphate stimulates the release of Ca^{2+} from intracellular stores, resulting in a first phase of Ca^{2+} elevation

(Gershengorn and Thaw, 1985). A second phase of Ca^{2+} lead to activation of a Ca^{2+} elevation is due to membrane depolarization, which leads to Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels (Li *et al.*, 1992). Increases in intracellular Ca^{2+} leads to the activation of a Ca^{2+} / calmodulin-dependent protein kinase (Jefferson *et al.*, 1991).

PKC activation and increased intracellular Ca^{2+} levels converge to activate the mitogen activated protein kinase (MAPK)-signaling pathway, also known as the extracellular regulated kinase (ERK) in GH3 pituitary tumor cells and subsequently activate PRL promoter (Ohmichi *et al.*, 1994; Wang and Maurer, 1999). However, our results showed that TRH has no effect on gfPRL promoter activity at a wide range of concentrations. It is contradictory to the report from Berwaer *et al.* (1993). We used similar experimental conditions and the only significant difference comes from the promoter. Our experiments used gfPRL promoter, while Berwaer was used rat PRL promoter. At present, there are neither *in vivo* nor *in vitro* studies on the effect of TRH on gfPRL synthesis and release in goldfish. As a result, TRH might have no effect on gfPRL gene transcription or its action might be prohibited due to the heterologous system.

In fact, TRH has different effects in different fish species. It reduces rainbow trout (*Oncorhynchus mykiss*) PRL synthesis and release in primary cultured PRL cells (Williams and Wigham, 1994), but stimulated the release of newly synthesized PRL in primary cultured pituitary cells of the common carp (*Cyprinus carpio*) (Kagabu *et al.*, 1998), suggesting that the different regulatory mechanisms employed in different fish species.

On the other hand, extensive investigations of TRH receptor signal transduction pathway in GH3 cells concluded that MAPK activation plays an important role of rat PRL promoter activation. However, the final mediator activated by MAPK pathway and stimulates rat PRL promoter is unclear, even though it is believed that Pit-1 is involved.

Furthermore, Wang and Maurer (1999) believed that pathways other than MAPK activation might also contribute to TRH effects on the PRL promoter. Recently, it has been reported that TRH stimulated phosphorylation of the epidermal growth factor (EGF) receptor in GH3 pituitary cells (Wang *et al.*, 2000). EGF is another factor that can stimulate rat PRL promoter (Berwaer *et al.*, 1993). The finding proposed a possibility of the involvement of other receptors in TRH's regulation of PRL promoter. Besides, the sequence of the rat PRL promoter is dissimilar to that of gfPRL. This means that the final mediator activated by TRH may not bind to the gfPRL promoter.

To further analyze the sequence, promoter deletion study was carried out. Interestingly, the deleted gfPRL promoter gave negative response to TRH. The results show that there is a possibility that the region between –560 and –405 bind the stimultatroy factors and the region between –405 and –248 binds the inhibitory factors.. Since TRH can activates different pathways and subsequenct activates different proteins. As a result, gfPRL promoter may response to the pathways that are different from rat PRL promoter does as they contain different sequences. Even though we have confined two regions of 156bp and 158bp long respectively, it is difficult to identity their corresponding binding factors. This is because the consensus sequence between mammalian and teleostean are not much conserved. Also, programs for searching potential transcription factors are not totally reliable. Therefore, to identify the binding factors, promoter deletion study and mobility shift assay should be carried out.

3.4 Conclusion

Pituitary-specific expression of gfPRL gene was clearly demonstrated by transient transfection assay in different mammalian cell lines. The gfPRL promoter showed transcription activity in GH3 cell only, which is a rat pituitary cell. Besides, the promoter is

negatively regulated by both DA in GH4ZR7, a rat pituitary cell line, and no effect upon TRH stimulation was found in this gfPRL gene promoter.

Promoter deletion study showed that DA inhibited gfPRL promoter transcription, most probably through inhibiting Pit-1 activity. While TRH showed inhibitory activity in the deleted promoter, the mechanism remains to be elucidated.

Chapter Four

Seasonal Study on gfPRL and gfGH expression

4.1 Introduction

Goldfish are among the most popular aquarium fish, and as such, extremely valuable both commercially and in serving as a model to study fish physiology. In addition, they are commonly used as bait fish, as live feed in fish production facilities, and for algal and plant control of small ponds (Robison and Buchana, 1988). As a result, the understanding of the endocrinology of reproduction and growth is important in order to improve the yield of goldfish.

GH and PRL are recognized as key hormones for growth and reproduction in mammal as well as in fish (Shinha, 1995). Many studies showed the variation in serum GH and PRL during the life cycle of fish. Moreover, both GH and PRL initiate their actions through interaction with their receptors. Therefore, to study the variation of GHR and PRLR expression will definitely provide an insight into the significance of the variation of serum GH and PRL level throughout the reproductive cycle.

4.2 Materials and Methods

4.2.1 Blood samples and radioimmunoassay

Goldfishs were purchased from Chong Hing Aquarium, Hong Kong. Blood samples (500-1000 μl) were taken from the caudal vasculature with a heparinized needle and syringe and centrifuged at 3,000Xg at 4°C. Plasma was stored at -20°C before use. Plasma GH levels were measured using a radioimmunoassay (RIA)(Wong *et al.*, 1992).

4.2.2 Preparation of ribonuclease free reagents and apparatus

General laboratory glassware was treated by baking at 180°C overnight. Items which were not susceptible for baking were treated with diethyl pyrocarbonate (DEPC, 0.1% v/v in ddH_2O). All solutions were treated with 0.1% DEPC overnight and then autoclaved.

4.2.3 Isolation of total RNA

The total RNA in tissue was isolated using TriPure Isolation Reagent (Roche). The procedures followed the instructions provided by the supplier. About 0.1 g of tissue was homogenized at room temperature with 1ml TriPure Isolation Reagent. Chloroform (200μl) was added into the homogenized tissue. The mixture was shaken vigorously for 10 sec and kept on ice. The mixture was centrifuged at 12,000 Xg for 10 min at 4°C in a microcentrifuge. The supernatant was saved into a fresh microcentrifuge tube immediately. The RNA was precipitated with 500μl isopropanol. The precipitated total RNA was collected by centrifugation at 12,000 Xg for 10 min at 4°C. The RNA pellet was washed by 1 ml 70% ethanol. Under this condition the RNA can be stored at -20°C for 1 year. Before use, the ethanol was removed by centrifugation. The total RNA pellet was dried in a DNA Speed Vac (Savant) for 10 min and then dissolved in 50 μl DEPC water. The yield and purity of RNA was determined by spectrophotometry (Section 2.2.2.3) and formaldehyde agarose gel

electrophoresis (Section 4.2.4).

4.2.4 Formaldehyde agarose gel electrophoresis of RNA

Total RNA or mRNA was fractionated on a 1% formaldehyde agarose gel. The agarose gel was prepared by mixing 0.5 g agarose, 10 ml 5X MOPS buffer (0.1M MOPS, 10mM sodium acetate and 5 mM EDTA•$2H_2O$) and about 30 ml distilled water. The mixture was boiled and then cooled down to around 60°C. Formaldehyde solution (12.3 M) of 2.7 ml was added to the mixture and finally added distilled water to fill up to 50 ml.

The gel was pre-run for 5 min at 50V before loading the samples. RNA sample (up to 30 μg in a 4.5μl volume) was mixed with 2.5 vol of RNA denaturing reagent (80% deionized foramide, 3.7 % formaldehyde, 1X MOPS) and 1/6 vol. of loading dye. The sample was incubated at 65°C for 15 min and ice chilled. After that, the sample was mixed with ethidium bromide (0.5 μg/ml) and loaded into the gel. A voltage of 40-60V was applied. The electrophoresis was stopped when the bromophenol blue has migrated to 2/3 length of the gel. The gel was examined under UV transillumination.

4.2.5 First strand cDNA synthesis

For gfPRL tissue distribution study, the first strand cDNA synthesis was performed by Superscript First Strand Synthesis System (GibcoBRL). The procedures were that provided by the supplier.

However, for gfPRLR and gfGHR RT-PCR, we used different system to generate first strand cDNA. The total RNA of 3 μg was mixed with 8 μl 5X Reaction Buffer (Promega), 10 units RNaseOut^{Tm} ribonuclease inhibitor (GibcoBRL), 10 pmole Oligo dT primer, 10 mM dNTP, 3 μl 0.1M DTT, 200 units M-MLV-RNase H minus (Promega) in 40 μl reaction for 1 h at 37°C. Finally, the reaction was terminated at 70°C for 15 min RNase H (Pharmacia

Amersham Biotech) of 5 units was added to each reaction mixture at 37°C for 30 min The reaction mixture containing first strand cDNA was stored at -20°C until use.

4.2.6 RT-PCR

For gfPRL tissue distribution study, two PCR reactions were performed for each template for gfPRL and β-actin respectively. For gfPRL RT-PCR, each reaction contained l μl cDNA, 10 pmoles gfFM (forward), 10 pmoles gfGCR (reverse), 1 μl 10 mM dNTP, 0.5 unit Taq polymerase, 5 μl 10X reaction buffer, and distilled water added up to 50 μl. For β-actin RT-PCR, the reaction composition was the same except the primers where 10 pmoles gfActinF and 10 pmoles gfActinR were used instead. The primers sequences were shown in Table 4.1.

The cycling reactions were performed on Robot Cycler 9600 (Stratagene).

94°C	2 min	
94°C	0.5 min	30 cycles
55°C	0.5 min	
72°C	1 min	
72°C	10 min	

The PCR products were analyzed in 1% agarose gel with TAE buffer.

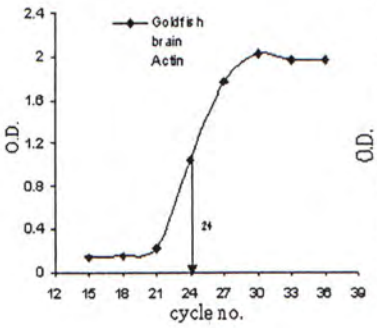
For gfPRLR and gfGHR RT-PCR, three separate PCR reactions were preformed for each template using three pairs of primers: gfActinF and gfActinR, GKF and GKR, and GfGH-SEQR1 and GfGH-SEQ950. Each reaction contained 2 μl cDNA, 10 pmoles forward primer, 10 pmoles reverse primer, 1 μl 10 mM dNTP, 0.5 unit Taq polymerase, 5 μl 10X reaction buffer, and distilled water added up to 50 μl. The cycling reactions were

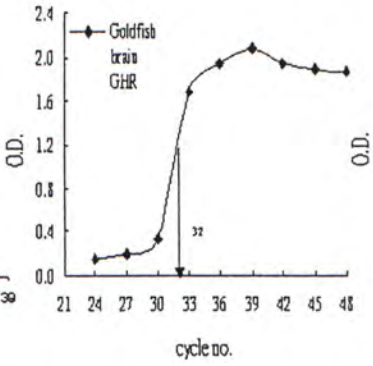
performed on a Perkin Elmer 9600 (ABI)

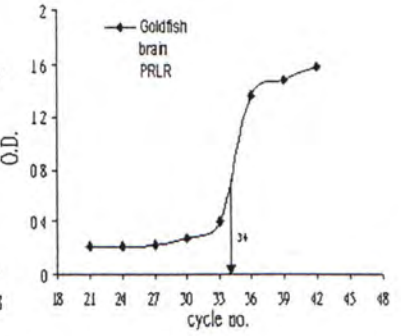
94°C	2 min	
94°C	1 min	The no. of cycles varied between different tissues and was shown in the table below
55°C	45 sec	
72°C	1 min	
72°C	10 min	

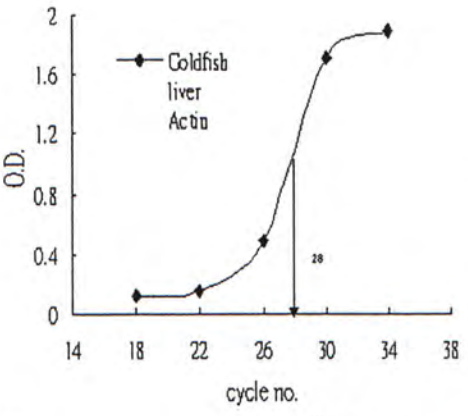
PCR cycles	Brain	Kidney	Liver	Testis	Ovary
gfβ-actin	24	23	28	29	33
gfPRLR	34	34	*	37	36
gfGHR	32	34	28	29	32

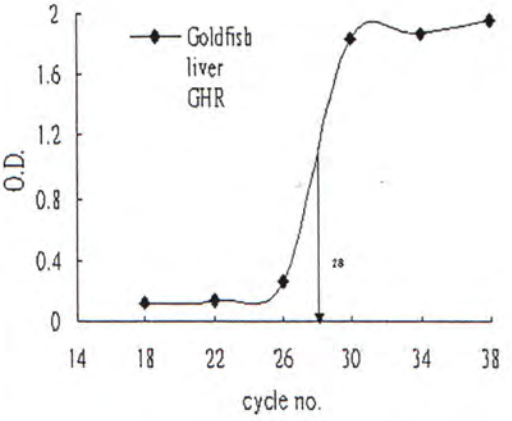
*gfPRLR transcript was not detected under 45 cycles


Goldfish brain Actin
O.D.
cycle no.


Goldfish brain GHR
O.D.
cycle no.


Goldfish brain PRLR
O.D.
cycle no.


Goldfish liver Actin
O.D.
cycle no.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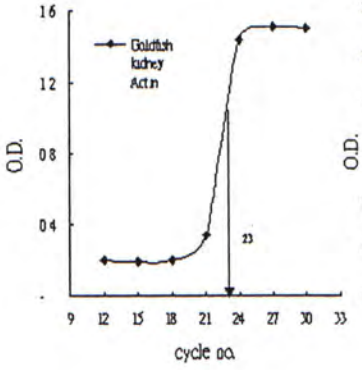
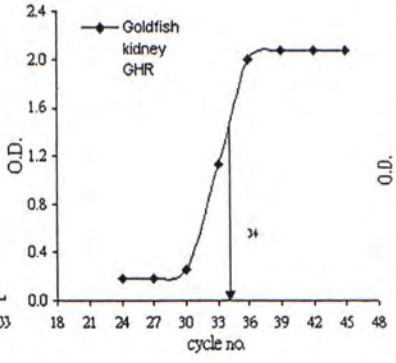
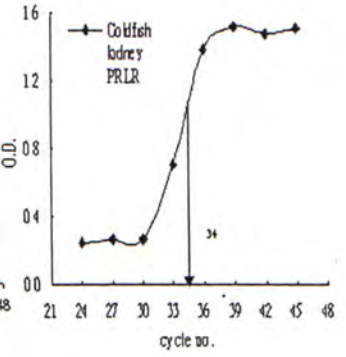
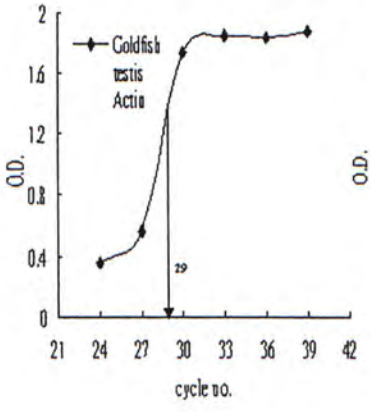
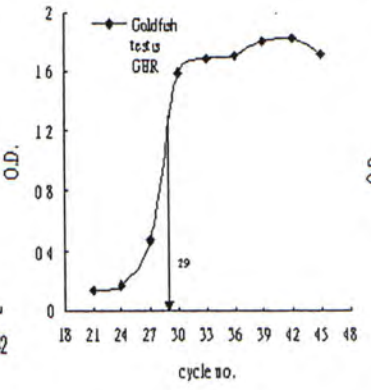
cycle no.

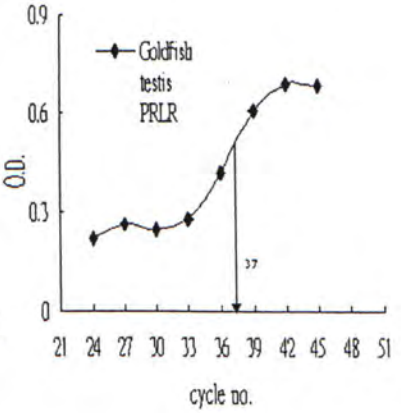

Goldfish kidney Actin
O.D.
cycle no.


Goldfish kidney GHR
O.D.
cycle no.


Goldfish kidney PRLR
O.D.
cycle no.


Goldfish testis Actin
O.D.
cycle no.


Goldfish testis GHR
O.D.
cycle no.


Goldfish testis PRLR
O.D.
cycle no.

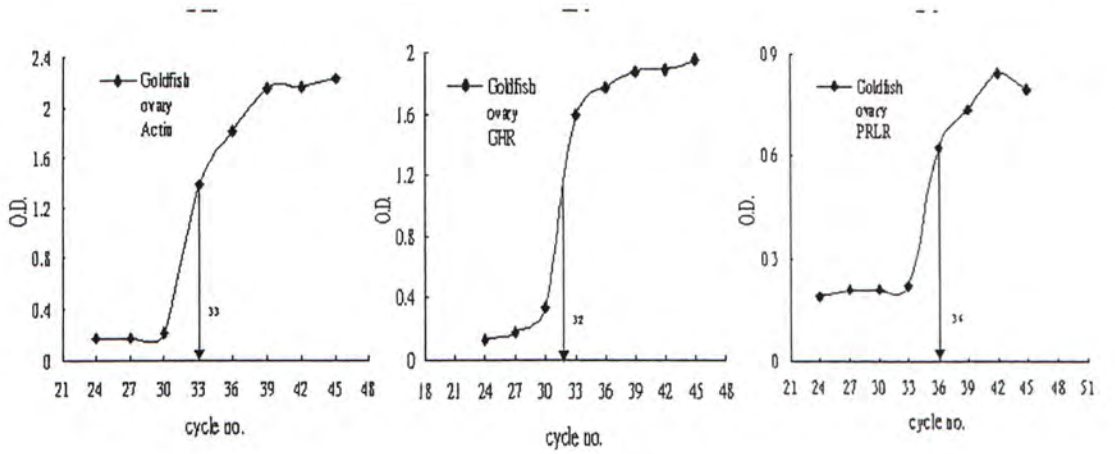


Fig.4.1. Validation of RT-PCR. Determination of valid PCR cycle numbers for RT-PCR detection of β-actin, PRLR and GHR in goldfish tissues: brain, liver, kidney, testis and ovary.

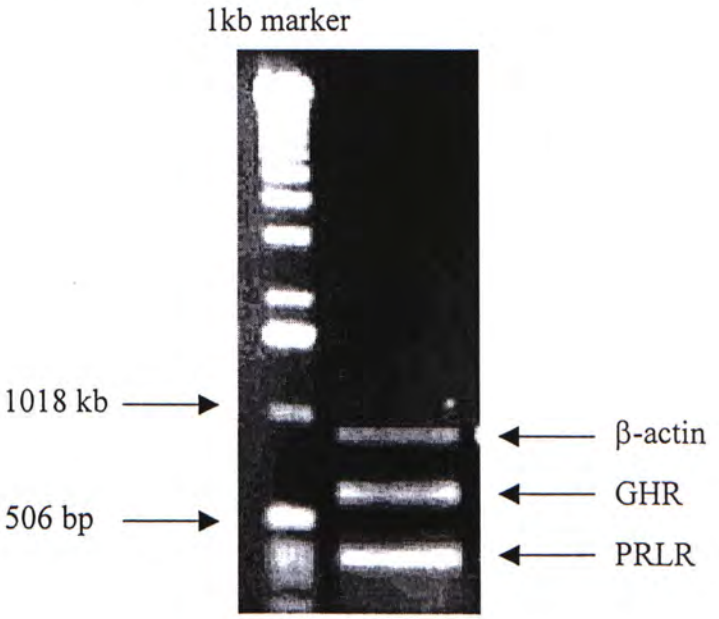
4.2.7 Analysis of RT-PCR

For gfPRL tissue distribution study, the PCR products were resolved in 1% agarose gel and stained with ethidium bromide solution. The PCR products were further analyzed by Southern blot as described in section 2.2.2.7.

For gfPRLR and gfGHR RT-PCR, the PCR products were resolved in 1% agarose gel and stained with ethidium bromide solution. Ten ul PCR product of each reaction of gfβ-actin, gfPRLR, and gfGHR from the same template were loaded into one well in order to obtain the relative light intensity of gfPRLR and gfGHR as shown in Table 4.1. To further eliminate the discrepancy due to staining efficiency in individual gel, equal amount of standard DNA marker was added in each gel, the light intensity of the 1kb DNA fragment of the DNA marker act as a reference for different gel. The light intensity of the each DNA fragments were measured by Molecular Imager (Bio-Rad GS505).

Table 4.1 Nucleotide sequences of oligonucleotide primers used in RT-PCR

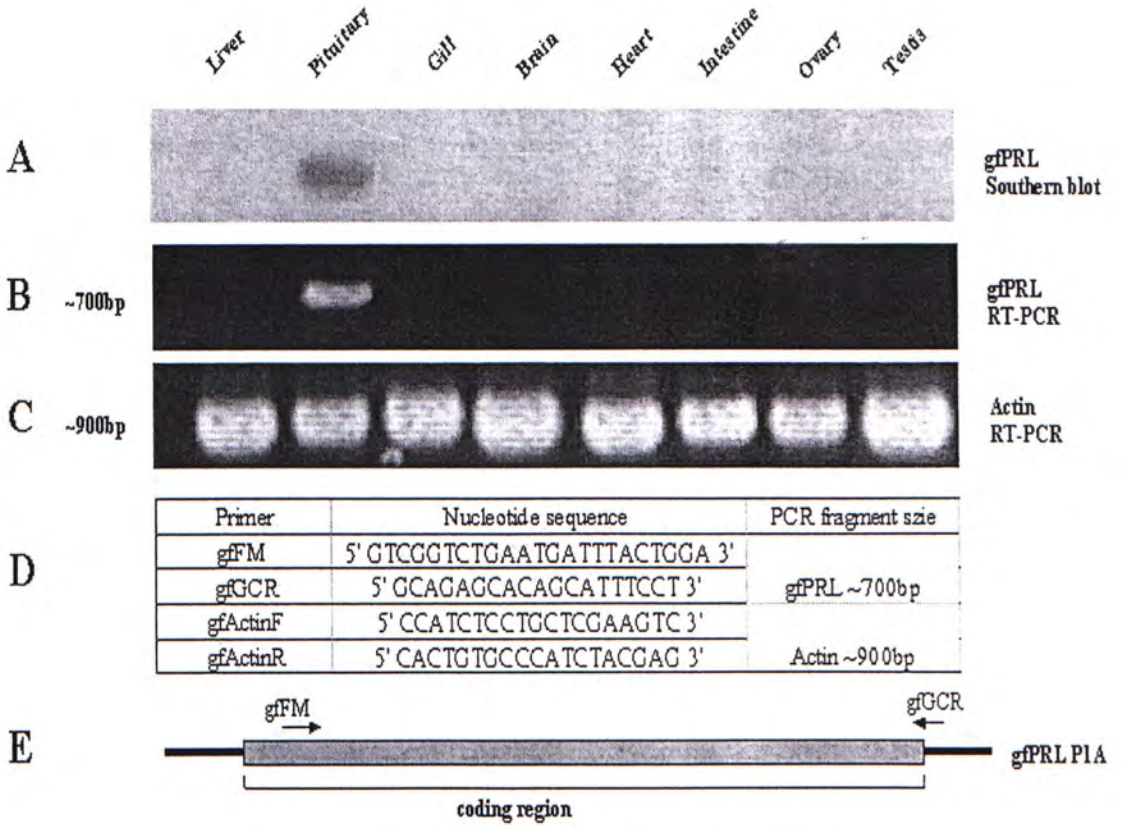
Primer name	Nucleotide sequence	PCR product
gfActinF	5' CCATCTCCTGCTCGAAGTC 3'	Actin ~900bp
gfActinR	5' CACTGTGCCCATCTACGAG 3'	
GKF	5' GGGTCAACTACAACATCACTGTGG 3'	PRLR ~370bp
GFR	5' AGGTGGTTTGGGTCCACTCACTCC 3'	
gfGHR-SEQR1	5' AATCACTGGTGGACACCAGGTACTC 3	GHR ~700bp
gfGHR-SEQ950	5' CGGACTTCTACCATGAGGAT 3'	



4.3 Results

4.3.1 Tissue-specific expression of gfPRL transcript

In adult seabeam (*Sparus aurata*) (Santos *et al.*, 1999), extra-pituitary PRL transcript was detected in the intestine, liver, ovary and testes by RT-PCR coupled with Southern blot analysis. In order to determine whether extra-pituitary PRL expression exists in goldfish, the tissue distribution of gfPRL was studied by RT-PCR coupled with Southern blot. The primers gfFM and gfGCR were designed at positions shown in Fig. 4.2E. A single product of the expected size (~1kb) was detected in the pituitary only. Southern blot analysis under high stringent conditions demonstrated that the RT-PCR product strongly hybridized with the radioactively labeled probe. The probe was generated by PCR using primers gfFM and gfGCR and gfPRL PIA cDNA as template. The 5'end of the PCR fragment was labeled with ^{32}P. The results (Fig.4.2) suggested that gfPRL transcript was confined to the pituitary only. The result in fact is consistent with the goldfish promoter transient transfection study.



Primer	Nucleotide sequence	PCR fragment szie
gfFM	5' GTCGGTCTGAATGATTTACTGGA 3'	gfPRL ~700bp
gfGCR	5' GCAGAGCACAGCATTTCCT 3'	
gfActinF	5' CCATCTCCTGCTCGAAGTC 3'	Actin ~900bp
gfActinR	5' CACTGTGCCCATCTACGAG 3'	

Fig.4.2. Comparative RT-PCR analysis of tissue distribution of gfPRL. Panel A: Southern blot analysis using ^{32}P labeled gfPRL P1A cDNA as a probe. Panel B: RT-PCR using gfPRL gene specific primers, gfFM and gfGCR. Panel C: RT-PCR using ß-actin primers. Panel D: Primer sequences and the expected size of PCR fragment. Panel E: Diagram shows the position of the gfPRL gene specific primers.

4.3.2 Sexual maturity of goldfish throughout the reproductive cycle

Gonadosomatic index (GSI) is the percentage of the gonad weight to the body wegith that reflects the sexual maturity of a fish. In female goldfish, GSI increased rapidly from September (0.01 ±0.2%) to November (1.6 ±0.5%) and remained stable until January (Fig.4.3). It rose to peak values in February and March (12.9 ±0.8%). After the spawning period, it sharply decreased in April (0.09 ±0.7%) and remained low until August (Fig 4.3).

In male goldfish, GSI started to rise gradually from September (0.005 ±0.07%) and reached peak level in February (4.8 ±0.4%). Then it decreased slightly in March (2.4 ±0.4%). After the spawning period, it stayed in the bottom from April to August (0.005 ±0.2%).

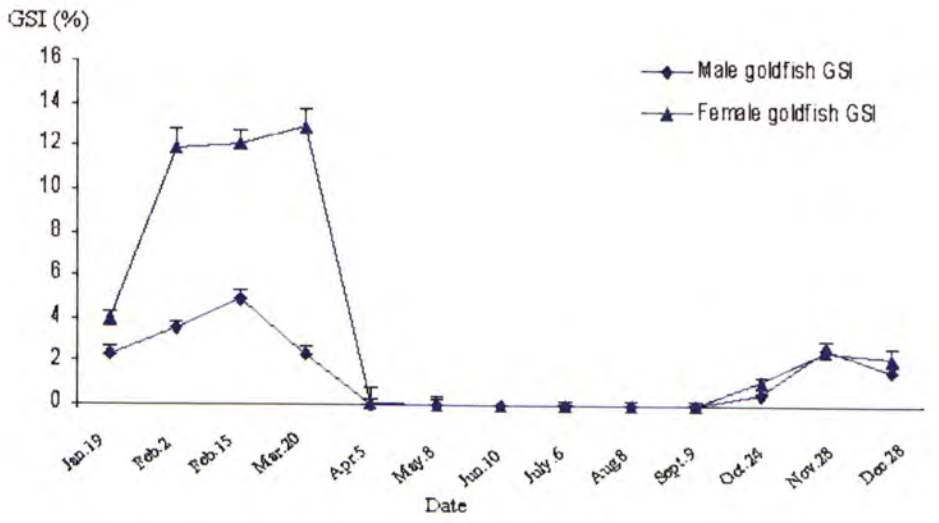


Fig.4.3. GSI of goldfish throughout the year. Data are from seven experiments and vertical bars represent SEM.

4.3.3 Serum gfGH level throughout the reproductive cycle

In male goldfish, the serum GH remained at low/basal level from January (19.4 ±1.5 ng/ml) to April (34.8 ±5.2 ng/ml)(Fig. 4.4). During the summer period, it rose significantly and the peak value was achieved in August (546.6 ±69.2 ng/ml). The serum GH decreased sharply and reached the basal level in October (24.8 ±2.6 ng/ml). From October to December, it remained at low level (Fig 4.4).

In female goldfish, the GH level was ranging from 10-20 ng/ml from January to April. It began to rise from May and the highest concentration (814.5 ±20.6 ng/ml) was recorded in July. Then it dropped obviously from August and remained at low level between September and December (16.9 ±1.1 ng/ml)

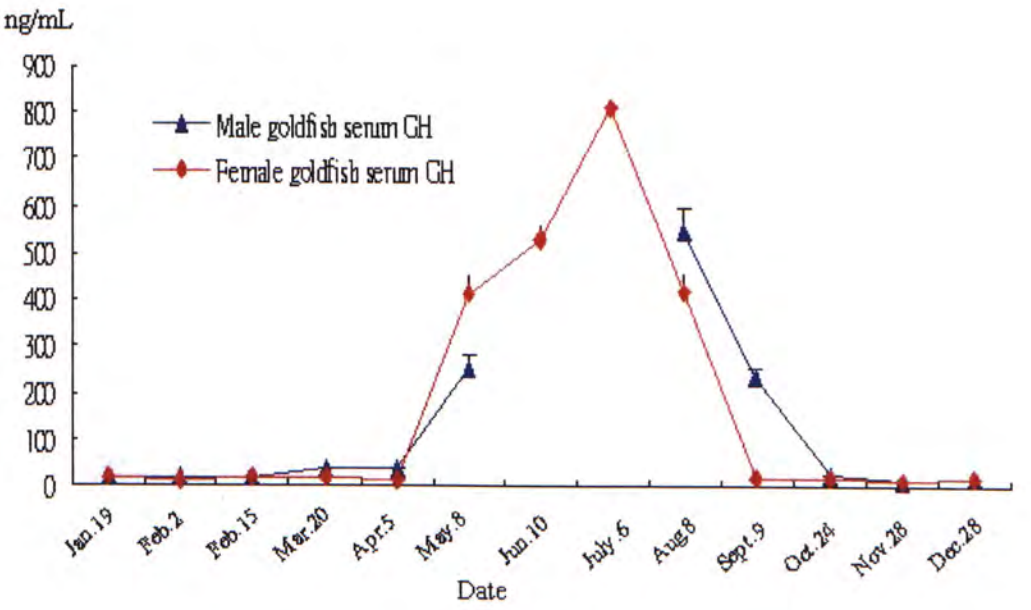


Fig.4.4. The serum GH levels throughout the year of 2000. Data are from five experiments and vertical bars represent SEM.

4.3.4 Serum gfPRL level throughout the reproductive cycle

In male goldfish, the serum PRL level was more or less the same within January (80.5 ±15.7 ng/ml) and June (78.9 ±25.2 ng/ml)(Fig. 4.5). It rose from July (112.8 ±20.3 ng/ml) and reached the peak value in August (221.3 ±9.3 ng/ml). It decreased gradually from September (164.5 ±4.9 ng/ml) to December (41.6 ±2.4 ng/ml), which was the lowest concentration within a year (Fig. 4.5).

In female goldfish, the serum PRL level rose slightly from January (55.3 ±3.5 ng/ml) to March (108.7 ±9.3 ng/ml). The second rise was observed from May and the peak value was in August (245.3 ±8.7 ng/ml). It began to decrease from September (171.4 ±6.3 ng/ml) to December (40.2 ±4.6 ng/ml).

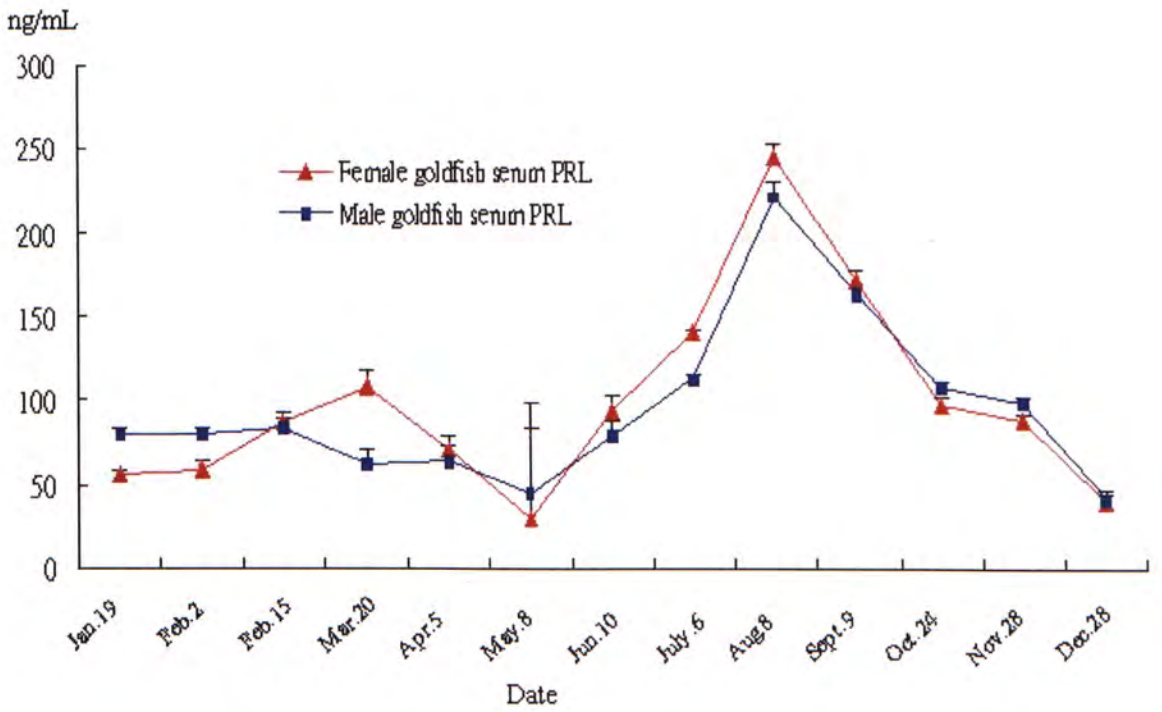


Fig.4.5. The serum PRL levels throughout the year of 2000. Data are from five experiments and vertical bars represent SEM.

4.3.5 The variation of gfGHR and gfPRLR mRNA in brain throughout the reproductive cycle

The variation of gfGHR and gfPRLR mRNA is showed in Fig. 4.6 and 4.7 respectively. The results demonstrated that no significant changes of gfGHR mRNA was detected throughout the year. Similar observation was showed in gfPRLR mRNA except for female goldfish. In female goldfish, gfPRLR mRNA level was high on January (0.29 ±0.01) and August (0.24 ±0.005) compared with other months.

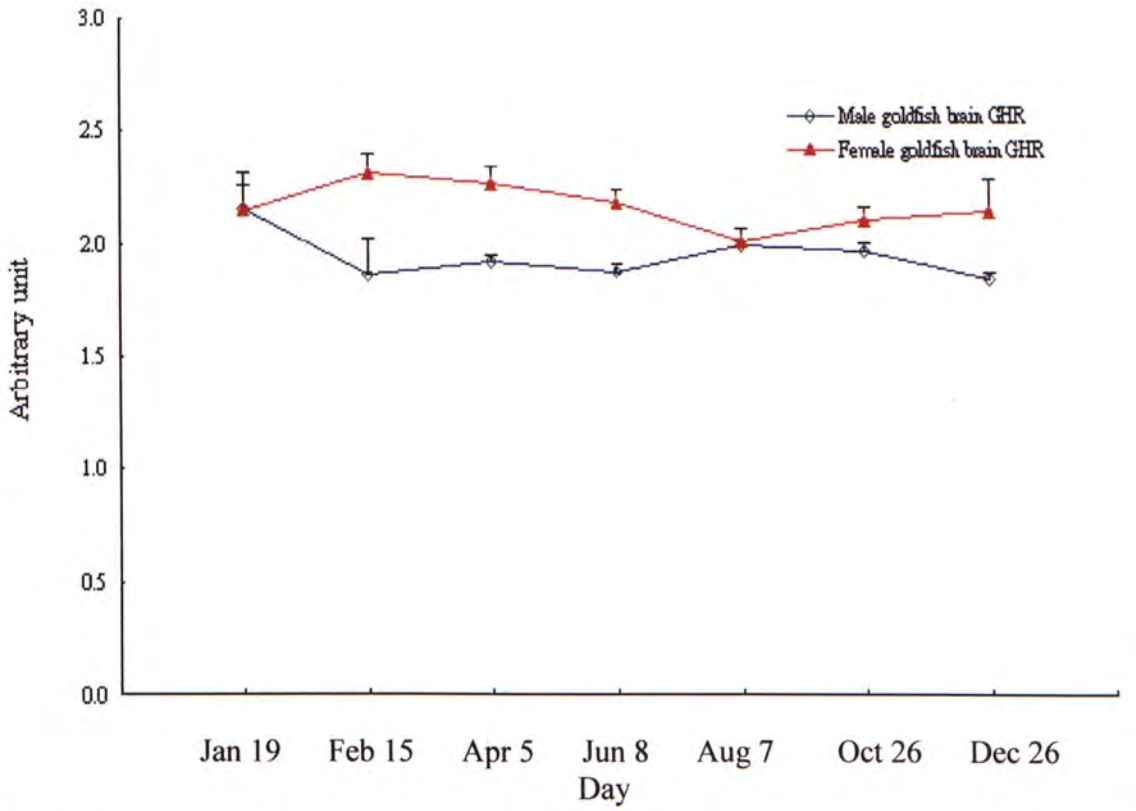


Fig. 4.6. Variation of gfGHR mRNA level in the brain throughout the year of 2000. Data are from five experiments and vertical bars represent SEM.

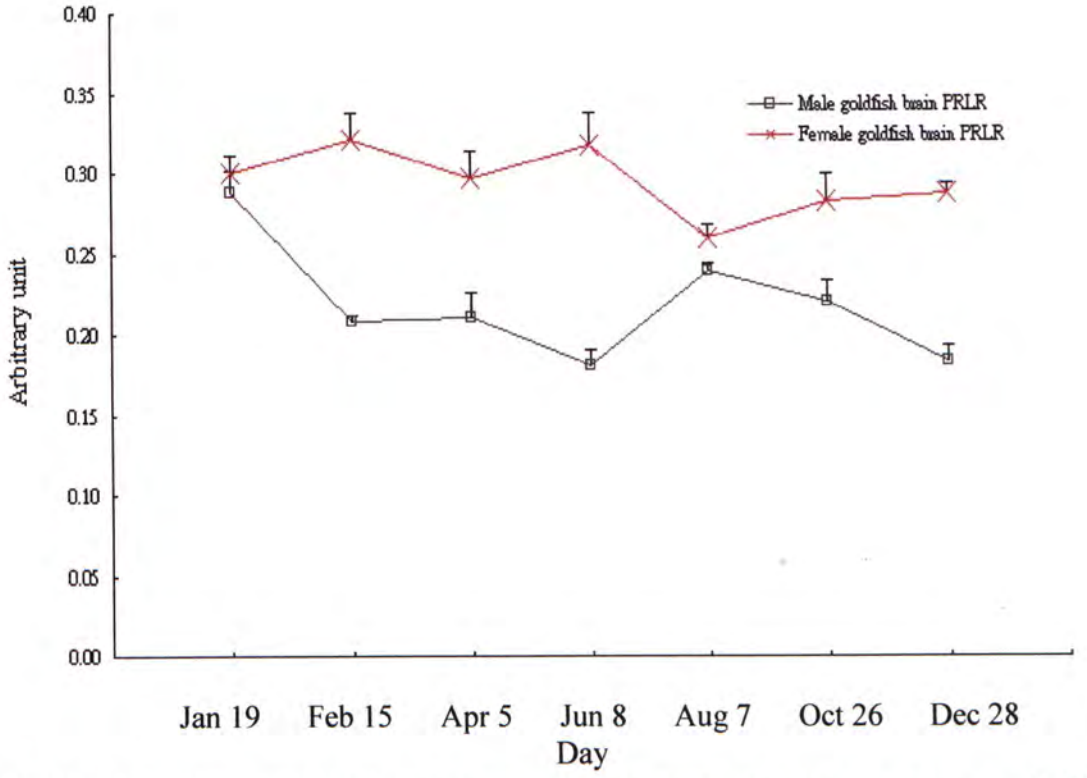


Fig. 4.7. Variation of gfPRLR mRNA level in the brain throughout the year of 2000.

Data are from five experiments and vertical bars represent SEM.

4.3.6 The variation of gfGHR mRNA in liver throughout the reproductive cycle

The variation of gfGHR mRNA is shown in Fig. 4.8. In male goldfish, gfGHR mRNA level rose from January (0.7 ±0.1) to February (0.9 ±0.1). It dropped dramatically until June (0.4 ±0.04) and remained stable in August (0.4 ±0.02). It rose again and reached highest level in October (1.2 ±0.01) and decreased sharply in December (0.4 ±0.005).

In female goldfish, gfGHR mRNA level was low in January (0.3 ±0.03) but rose significantly in February (1.4 ±0.1). Alternative rise and drop was observed in the following months: April (0.5 ±0.03). June (1.5 ±0.05), August (0.4 ±0.05), October (0.7 ±0.05) and December (0.3 ±0.02). Peaks of female gfGHR overlap with those of male gfGHR except in June. In male, gfGHR mRNA was low (0.4 ±0.04) in June while it is high in female (1.5

±0.05). Goldfish PRLR mRNA was not detected in the liver of whole year even we used 46 cycles PCR.

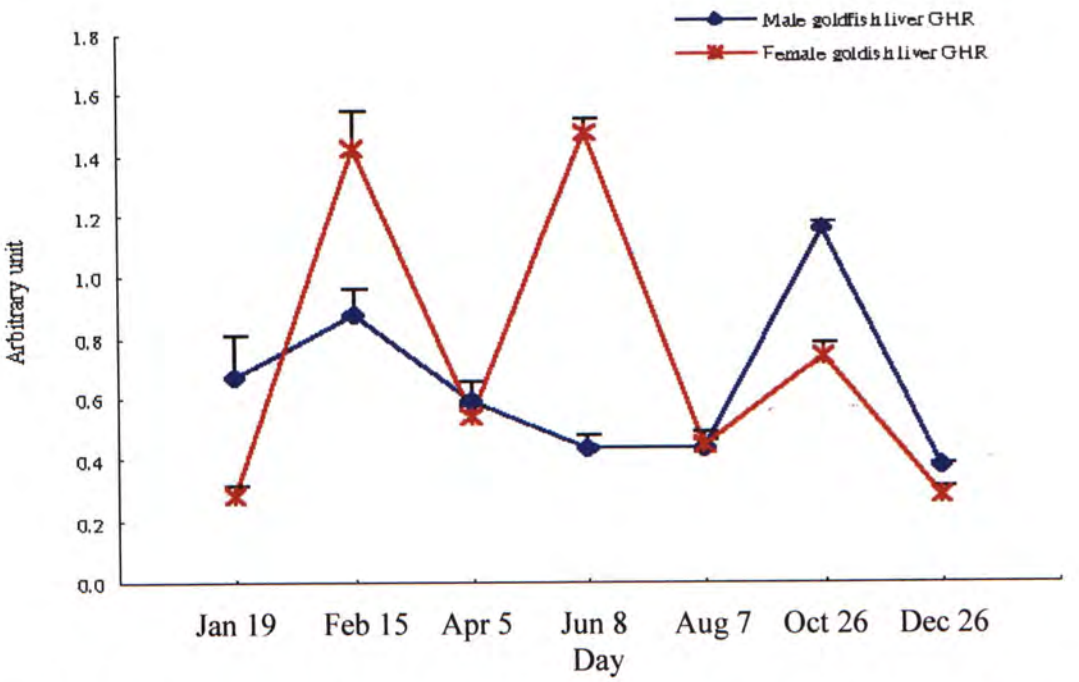


Fig. 4.8. Variation of gfGHR mRNA level in the liver throughout the year of 2000. Data are from five experiments and vertical bars represent SEM.

4.3.7 The variation of gfGHR and gfPRLR mRNA in the kidney throughout the reproductive cycle

The variation of gfGHR and gfPRLR mRNA is showed in Fig. 4.9. In both male and female goldfish, the highest gfGHR mRNA levels were recorded in February (Male, 1.1 ±0.1; Female, 1.4 ±0.05). It gradually decreased until November (Male, 0.4±0.04; Female, 0.7 ±0.1) and rose slightly from December (Male, 0.9 ±0.1; Female, 1.2 ±0.2).

Similar results were obtained in gfPRLR mRNA level of both male and female goldfish. The peak values were observed in February (Male, 1.2 ±0.05; Female; 1.4 ±0.04). In male, gfPRLR mRNA decreased from March (0.9 ±0.02) to December (0.4 ±0.02). In female, a

similar phenomenon was observed except there is a little rebounce in November (0.9 ±0.04).

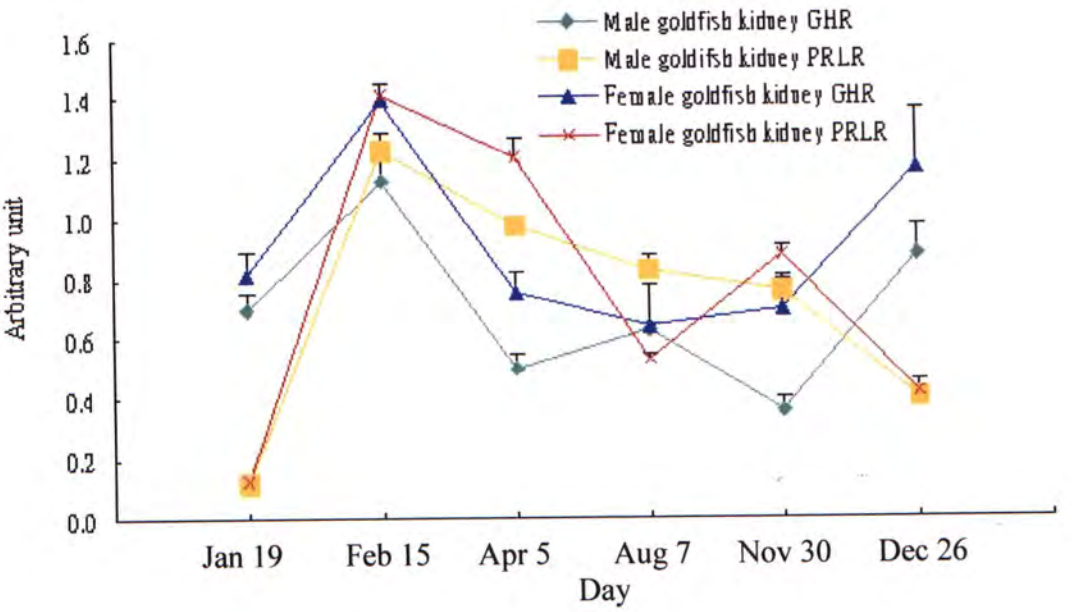


Fig. 4.9. Variation of gfGHR and gfPRLR mRNA level in the kidney throughout the year of 2000. Data are from five experiments and vertical bars represent SEM.

4.3.8 The variation of gfGHR and gfPRLR mRNA in the gonads throughout the reproductive cycle

The variation of gfGHR mRNA in ovary and testis is showed in Fig. 4.10. In testis, gfGHR mRNA rose from January (1.6 ±0.2) to peak level in February (2.2 ±0.2). It decreased gradually and reached lowest value in Jun (0.3 ±0.1). It rose again from August (0.9 ±0.1) to December (1.4 ±0.1).

In ovary, the peak value also existed in February (1.2 ±0.2). Besides, the lowest mRNA level was detected in Jun (0.2 ±0.02). However, the variation between August and December was different from that observed in testis. It rose in August (0.8 ±0.06) and decreased in the following months.

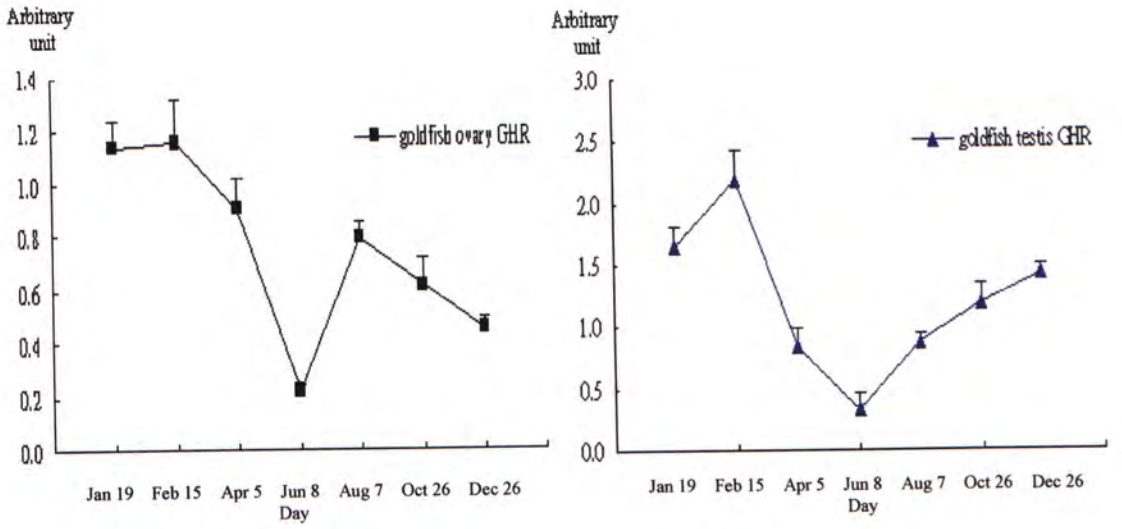


Fig. 4.10.Variation of gfGHR mRNA level in the gonads throughout the year of 2000. Data are from five experiments and vertical bars represent SEM.

The variation of gfPRLR mRNA in ovary and testis were showed in Fig. 4.11. Interestingly, gfPRLR mRNA was only detected in January, February and April in either ovary or testis. No significant changes were observed among these three months.

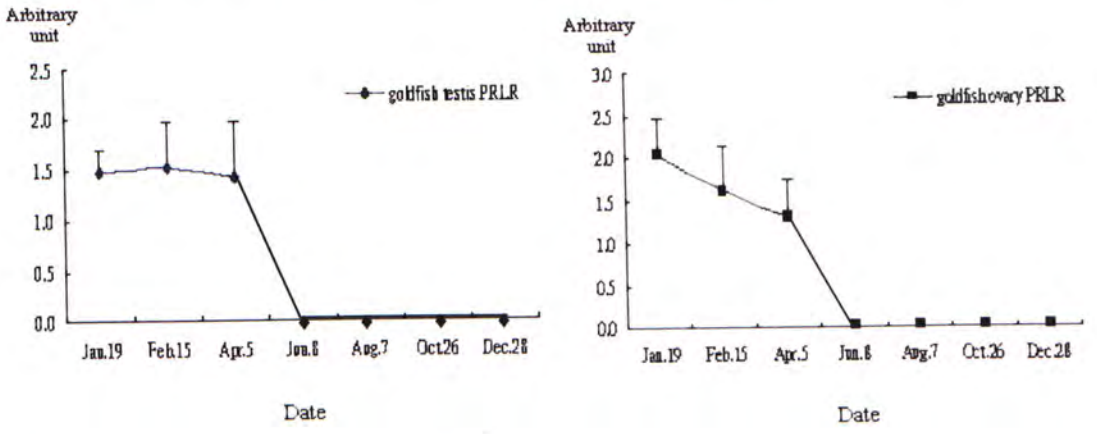


Fig. 4.11. Variation of gfPRLR mRNA level in the gonads throughout the year of 2000. Data are from five experiments and vertical bars represent SEM.

4.4 Discussion

4.4.1 Tissue-specific expression of gfPRL transcript

RT-PCR coupled with Southern blot was used rather than Northern blot to detect the gfPRL mRNA level in different tissues. It is because RT-PCR has a much higher sensitivity and can detect a low copy number of transcripts. The results show that gfPRL transcript was only detected in the goldfish pituitary but not in other tissues. The expression of the PRL gene in tissues other than pituitary has not previously been demonstrated in teleost except in seabream (Santos *et al.*, 1999), although it is well-established in other vertebrates (Jonathan *et al.*, 1996). In sea bream, extrapituitary PRL was detected in intestine, liver, ovary and testes. The function of extrapituitary PRL in seabream remains unknown. However, there is no result concerning the gfGH tissue specific expression even though it is believed to be pituitary specific.

On the contrary, Yang *et al.* (1999) have found that GH family protein genes (GH, PRL, and SL) are expressed in the developing embryos prior to the formation of the pituitary gland in rainbow trout. PRL mRNA was detected only in the head part of the fry whereas GH and SL mRNA were detected in both the head and trunk. In adult animals, though high levels of GH mRNA were primarily detected in the pituitary gland, brain, gill, and heart, low levels of GH mRNA were also detected in the kidney, liver, pyloric ceaca, and ovary. Results of the current study and those reported by Yang *et al.* (1997) suggested that GH and SL genes are also expressed in extrapituitary tissues even after the organogenesis of the pituitary gland. It was suggested that these hormones might play important roles yet to be identified during embryonic development in fish.

4.4.2 Sexual maturity of goldfish throughout the reproductive cycle

The GSI represents the sexual maturity of a fish. The results showed that goldfish

become sexually matured from February to March. They became sexually regressed from April to August. On September, the sex organ began to develop again. The period from September to sex maturation is called recrudescence.

Goldfish are multiple spawners and they can spawn at water temperatures as low as 12-13°C.

4.4.3 Serum gfGH and gfPRL level throughout the reproductive cycle

Seasonal variations in the rate of body growth appear to be universal in teleost species found in temperate climatic zone (Ricker, 1979). Variations in somatic growth rates throughout the year have been described for several teleost species, including the common carp *Cyprinus carpio*, (Kawamoto *et al.*, 1957), the suckers *Catostomus catostomus* and *C. commersoni* (Basset, 1957), the barbell *Barbus barbus*, (Hunt and Jones, 1975), the black crappie *Pomoxis nigromaculatus*, (Haines, 1980), the perch *Perca fluviatilis* and *P. flavescens* (Le Cren, 1951; Kearns and Atchison, 1979), the northern pike *Esox lucius*, (Diana and Mackay, 1979), the brown trout *Salmo trutta*, (Swift, 1961), and various coregonid species (Hogman, 1968; Hagen, 1970; Berg, 1970), and several marine teleost species from the Azov and Black Seas (Shul'man, 1974). In all these species, somatic growth tends to follow the seasons, with the highest growth rate occurring in the summer and a reduced rate of growth in the winter.

Food availability, reproductive activities, and environmental parameters such as temperature and photoperiod undoubtedly contribute to seasonal variations in growth rates(Brett, 1979; Swift, 1961; Shul'man, 1974). Studies have examined the role of the endocrine system in the somatic growth cycle in fishes, it was suggested that seasonal variations in GH contributed to the growth cycle (Marchant and Peter, 1986; Kayes, 1977; Adelman, 1977). Our result showed that GH level was high only during the summer period (May to August). There is no evidence to show any consistent relationship between water

temperature and GH secretion. However, it was showed that GH level correspond well with photoperiod (Bjornsson *et al.*, 1994; 1995; 2000; Marchant and Peter, 1986). Decreased day length will suppress the serum GH level in fish (Bjornsson *et al.*, 2000).

Much less study concern the role of teleost PRL in the reproductive cycle. The variation of plasma PRL is more or less coincided with GH. It will be more interesting as we considered the antagonistic relationship of PRL and GH in osmoregulation. This implies that GH and PRL share similar seasonal actions e.g. growth in teleost. However, there is still an unanswered question: why PRL varies seasonally in a more or less constant environment? This suggests that there is another critical function exist in teleost or the variation of PRL is to balance the changes of GH level in plasma in order to allow a fish to live in an osmotic constant environment. In either case, we should study the interaction between GH and PRL expression.

4.4.4 The variation of gfGHR and gfPRLR mRNA in the brain throughout the reproductive cycle

The function of GH in hypothalamus was clearly demonstrated by Minami *et al.*, (1993). GH regulates the expression of somatostatin (SS) and GRF in hypothalamus. Apart from its effects on the hypothalamus, it is also well established that GH stimulates neuronal and glial proliferation and maturation, and increases brain size in rats. Also, the hormone exerts important neuromodulatory actions in discrete brain regions and participate in many central functions such as modulation of feeding behavior, sleep and breathing control, and learning and memory in laboratory animals (Noguchi, 1996; Harvey *et al.*, 1993). Regarding the role of GH in reproduction, Chandrashekar *et al.* (1999) found that the basal plasma LH level was not affected, the LH as well as the testosterone response to GH treatment was significantly decreased in GHR knockout mice.

On the other hand, scientists identified numerous deficiencies in PRLR knockout mice. Female homozygous mice are completely infertile and lack normal mammary development, while hemizygotes are unable to lactate following their first pregnancy. PRLR knockout males and females have markedly elevated (30- to 100-fold) serum PRL levels and in some instances pituitary hyperplasia is present. Maternal behavior is severely affected in both hemizygous and heterozygous animals (Kelly *et al.*, 2001).

In this study, we found that both gfGHR and gfPRLR mRNA level remained constant throughout the whole year. This also suggests that there is no consistent relationship between plasma GH and PRL and the concentration of GHR and PRLR mRNA in the brain. However, there may be some variation in certain cell types (e.g. in hypothalamus), but this is masked by the amount of GHR and PRLR mRNA in other cell types. As a matter of fact, similar findings were observed in chicken that there is no consistent relationship between plasma PRL and reproductive state (Ohkubo *et al.*, 1998).

4.4.5 The variation of gfGHR mRNA in the liver throughout the reproductive cycle

Hepatic GHR have been found to be either up- or down-regulated by plasma GH in mammals (Gluckman and Breier, 1989). In teleost, regulation of hepatic GHR by high plasma GH has also been reported (Gray *et al.*, 1990; Mori *et al.*, 1992; Hirano, 1991). Our results clearly demonstrated that gfGHR mRNA level negatively correlated with the serum gfGH level in male goldfish fish. It has been suggested that the reduced gfGHR mRNA level during summer period may be due to the metabolic status as the high water temperature may reduce food ration(Gray *et al.*, 1992; Duan and Hirano, 1992). While in female, the relationship between serum GH and hepatic GHR is unclear, and their levels showed regular variation with a 4-month cycle during the reproductive cycle. The role of such variations in female teleost remains to be elucidated.

The hepatic PRLR was not detected. These observation was consistent with the previous published report (Tse *et al.*, 2000). The PRLR in the goldfish liver was very low and which can only be detected when RT-PCR is coupled with Southern blotting. Based on our result, we believe that the role of PRLR in liver is much less significant than GHR.

4.4.6 The variation of gfGHR and gfPRLR mRNA in the kidney throughout the reproductive cycle

Kidney is one of the major osmoregulatory organs in fish. The variation of the expression of both renal GHR and PRLR mRNA is significant, as we know kidney is one of the major organs for osmoregulation. However, under the stable osmotic environemt, it is really hard to give a reasonable explaination for such variation.

4.4.7 The variation of gfGHR and gfPRLR mRNA in the gonads throughout the reproductive cycle

Study of the changes in GHR in rainbow trout testicular tissue during a spermatogenetic cycle showed that relatively large numbers of GHR were present in the immature trout testis, that is, before any sign of spermatogenetic development could be detected (Gomez *et al.*, 1998). At that stage, somatic cells and stem germ cells (or A spermatogonia) are believed to proliferate as the gonad grows slowly.

GHR was maximum at the beginning of spermatogenetic development (B spermatogonia proliferation), decreased progressively but markedly during the advance of meiosis, and became minimal at the end of spermatogenesis. This study suggested that GH could be potentially important for gonadal functions during the first steps of spermatogenesis. To compile with our study, we found that the GHR mRNA level was highest during the period of gonadal development and remained at low level during recrudescence and sexual regression period. It obviously demonstrated that GH was

essential for gonadal functions.

In hypophysectomized male rats, exogenous GH administration improved testicular steroidogenic responses to LH and LH receptor content (Zipf *et al.*, 1978) and increased IGF-1 mRNA content (Closset *et al.*, 1989) and IGF-1 peptide accumulation (D'Ercole *et al.*, 1984) in the testis. Furthermore, GH stimulation of IGF-1 mRNA content in Leydig/interstitial cell culture (Lin *et al.*, 1990) and IGF-1 peptide accumulation in Sertoli cell culture (Tres *et al.*, 1986) were also reported. These results suggested that IGF-I might be an important modulator/mediator of GH action in the testis. In teleost, GH treatments are able to modify, *in vivo* and *in vitro*, the production of sexual steroids at the gonad level in teleost fish (Singh and Thomas, 1993; Van der *et al.*, 1990; Singh *et al.*, 1988b; Le Gac *et al.*, 1992) and to modify testicular IGF-I mRNA level in rainbow trout (Le Gac *et al.*, 1996b).

To further understand the mechanism of GH on gonadal function, the distribution of the GHR in the different cell populations in the testis must be known. Lobie *et al.* (1990b) reported a strong GHR/GHBP immunoreactivity in Leydig and Sertoli cells and moderate activity in most rat germ cell types, while in human testis, *in situ* hybridization revealed that GHR mRNA expression is mainly in the seminiferous epithelium (Mertani *et al.*, 1995). Besides, the presence of GHR in Sertioli cells also strongly suggested as the marked reduction of GHR concentration during the reproductive cycle was compatible with the decreased proportion of Sertoli cells during fish spermatogenesis (Billard, 1983). Therefore, GH could act directly on the testis, not only by interfering with the interstitial steroidogenic cells, but also at the level of the germinal epithelium. In previous studies, it was found that trout Sertoli cell express IGF-I mRNA (Le Gac *et al.*, 1996a) and that IGFs can directly stimulate spermatogonial multiplication *in vitro* (Loir, 1994; Loir and Le Gac, 1994). Furthermore, rainbow trout GH has a stimulatory effect on *in vitro* spermatogonia and

spermatocyte multiplication when testicular somatic cells (Sertoli cells plus interstitial cells) are present (Gomez *et al.*, 1998), while it has no effect in the absence of these cells (Loir, 1994). As a result, GH may promote tubular and Sertoli cell maturation, and provide new information for the interpretation of the beneficial effect of GH in the treatment of male infertility (Shoham *et al.*, 1994).

On the other hand, Eckery *et al.*, (1997) localized the mRNA encoding GHR in oocytes of preantral follicles in the sheep. Strong GHR immunoreactivity has also been shown in scattered oocytes of rat ovaries (Lobie *et al.*, 1990a). The results of the *in situ* hybridization and immunohistochemical studies implied that the GHR plays an important role especially in the early stages of follicalogenesis. This is supported by the finding that administration of exogenous GH to cows significantly increased the number of small follicles (Gong *et al.*, 1991). Additionally, application of GH raised follicle diameter in *in vitro*-perfused rabbit ovaries in a dose-dependent manner (Yoshimura *et al.*, 1993).

Kolle *et al.*(1998) found that the GHR transcript is already synthesized in primordial follicles before birth supports the concept that GHR is involved in the development and differentiation of primordial follicles both in prenatal and in postnatal life. All secondary and tertiary follicles in the fetal ovary, which degenerate after birth, expressed neither the mRNA for GHR nor the receptor protein. Thus, GH might take part in the initiation of the further development of the primordial oocytes after birth. The role of GH during this complex process remains to be evaluated.

Rubin and Specker (1992) have demonstrated that homologous PRL can induce secretion of testosterone in minced testes of courting (bachelor) tilapia. Treatment with recombinant salmon GH significantly increased plasma concentrations of testosterone in males and estradiol-17 beta in females; purified chum salmon PRL had similar effecteds on

testosterone levels in males. Further, treatment with these hormones prevented the decline in gonadal weight observed after hypophysectomy in both males and females (Singh *et al.*, 1988b). It, therefore, demonstrated that purified chum salmon PRL possessed steroidogenic and gonadotropic activities. However, the significance of these effects of teleost PRL is not known. More experiments are needed to further elucidate the role of PRL on gonad function.

4.5 Conclusion

The results demonstrated that gfPRL is specifically expressed in the pituitary. Its secretion is seasonally regulated, of which the rhythm concides with gfGH secretion. Both gfGH and gfPRL hormones secretion lag behind the sexual maturity process. The mRNA levels of both receptors of gfGH and gfPRL expressed only during the sex mature period, at which both serum gfPRL and gfGH level are very low. It strongly implies the negative coorelation between plasma GH and PRL level and gonad GHR and PRLR mRNA level. The significance remains to be elucidate.

Chapter Five

Production of Recombinant gfPRL

5.1 Introduction

In teleost fish, PRL is involved in several important physiological processes such as growth, reproduction, metabolism and mucus production; the hormone's primary role being regulation of water and electrolyte homeostasis (Bole-Feysot *et al.*, 1998). To investigate the function of fresh water teleost PRL other than osmoregulation, and its interaction with gfPRLR, large amount of gfPRL is required. However, isolation of native gfPRL from pituitary is impractical because of its expensive cost, lengthy processing time and low production yield. As a result, production of recombinant gfPRL is needed.

In the past decade, systems for expression of recombinant proteins developed rapidly. A wide range of expression systems, host organisms, and processing procedures has been described. Amongst these, *Escherichia coli* remains an important organism for production of recombinant proteins in both laboratory scale and industry production. This is because only a few organisms can satisfy all the criteria, such as high growth rate, relatively simple nutritional demands, genetic stability, ease of product purification, and appropriate posttranslational modification. In addition to the advantage of genetic manipulation being a straightforward process in *E. coli*, this organism has the ability to accumulate many proteins to at least 20% of the total cellular protein and to translocate them from the cytoplasm to the periplasm. This makes *E. coli* an attractive host for large scale production of recombinant proteins in this organism presents advantages of many bioprocessing strategies. However, since *E. coli* is prokaryotic cells, improper or even no posttranslational modification is

introduced to the recombinant protein, and formation of inclusion bodies occurs frequently, which is comparable to the yeast expression system (eukaryotic expression system). It implied that an extra refolding step is necessary to produce biological active recombinant proteins. However, the purification process is simplified as the inclusion bodies already contained over 90% of recombinant protein. All in all, *E. coli* is a*n* efficient and cost-effective host for recombinant protein expression. Swenen et al (1991) succeeded in expressing biological active tilapia PRL of both isoforms (tiPRL-I or tiPRL-II) in *E. coli* (Swennen *et al.*, 1991) to study the different role of both isoforms *in vitro.*

The expression construct, pRSETA, containing the gfPRL P8A cDNA sequence was expressed in *E. coli.* We succeeded in expressing the recombinant gfPRL and it was purified and refolded. The activity of the recombinant gfPRL was proved by luciferase assay in CHO cells, which are stably transfected with gfPRLR expressing construct. This large amount of functional recombinant gfPRL is ready for further investigation and characterization of the physiology of gfPRL by, say, receptor binding assay and RIA etc .

5.2 Materials and Methods

5.2.1 Buffers and Reagents

Blocking Solution

Skim milk powder	0.5g
1X TTBS	10ml

Freshly prepare before use

Carbonate-bicarbonate buffer for ELISA

Na_2CO_3	1.59g/L
$NaHCO_3$	2.93g/L
NaN_3	0.2g/L

Adjust to pH 9.6

Coomassie Blue Stain

Coomassie Brilliant Blue	0.05%(w/v)
Acetic Acid	10%(v/v)
Methanol	40%(v/v)
Distilled water	50%(v/v)

Store at room temperature

Destaining Solution for SDS-PAGE

Glacial acetic acid	10ml
Methanol	40ml
Distilled water	50ml

Store at room temperature

Developing Solution for Western blot

Tris-HCl, pH9.0	0.1M
$MgCl_2$	5mM
BCIP	0.15mg/ml
NBT	0.3mg/ml

The solution should be freshly prepared.

Electrode Buffer, 5X for SDS-PAGE

Tris Base	(50mM) 6g
Glycine	(2.6M) 28.8g
SDS	(0.1%) 1g
Distilled water	mix up to 1L

Store at room temperature

Equilibration Solution A for Size Exclusion Chomatography

Ammonium bicarbonate	50mM
Distilled water	800ml
Adjust to pH8.0	
Distilled water	mix up to 1L

Filtered the solution with 0.2μm membrane and stored at room temperature.

Equilibration Solution B for Anion Exchange Chomatography

Ammonium bicarbonate	50mM
NaCl	1M
Distilled water	800ml
Adjust to pH7.6	
Distilled water	mix up to 1L

Filtered the solution with 0.2μm membrane and stored at room temperature.

Extraction Solution

Ammonium bicarbonate	50mM
EDTA	5mM
PMSF	15mM
Distilled water	800ml
Adjust to pH8.0	
Distilled water	mix up to 1L

Filtered the solution with 0.2μm membrane and stored at room temperature.

Elution Solution for anion exchange chomatography

Ammonium bicarbonate	50mM
Distilled water	800ml
Adjust to pH7.6	
Distilled water	mix up to 1L

Filtered the solution with 0.2μm membrane and stored at room temperature.

Lower Buffer

Tris Base	(1.5M)18.15g
Distilled water	mix up to 100ml

Adjust the pH to 8.8 by HCl.

Store at 4°C

PNPP substrate solution for ELISA

p-nitrophenyl phosphate	10mg/ml
Tris buffer	0.2 M

The solution should be freshly prepared

Protein Sample Buffer, 2X

Upper buffer	1ml
Glycerol	1ml
10% SDS	1ml
2-ME	0.1ml
0.05% Bromophenol blue	0.2ml
Distilled water	mixed up to 8ml

Store at 4°C

Renaturing Solution

β-mercaptoethanol	5mM
EDTA	2mM
GSH	5mM
GSSG	0.5mM
NDSB-256	1M

TBS (1X) instead of distilled water was used as a solvent

5% Stacking Gel for SDS-PAGE

Upper Buffer	0.75ml
30% Acrylamide (30:0.8)	0.5ml
10% SDS (w/v)	0.03ml
10% APS (w/v)	0.04ml
TEMED	3ul
Distilled water	1.65ml

10% APS should be freshly prepare and TEMED should be added last

15% Separating Gel for SDS-PAGE

Lower Buffer	1.25ml
30% Acrylamide (30:0.8)	2.5ml
10% SDS (w/v)	0.025ml
10% APS (w/v)	0.075ml
TEMED	1.25ul
Distilled water	1.15ml

10% APS should be freshly prepare and TEMED should be added last

TBS, 10X

Tris Base	(200mM) 2.42g
NaCl	(8%) 80g
Distilled water	800ml
Adjust to pH8.0	
Distilled water	mix up to 1L

Store at room temperature

TBST for ELISA

10X TBS	100ml
Tween 20	1ml
Distilled water	mix up to 1L

TTBS, 1X, for Western blot

Tris Base	(20mM) 0.242g
NaCl	(0.8%) 8g
Tween 20	(0.1%) 1ml
Distilled water	mix up to 1L

Store at room temperature

Transfer Buffer, 1X, for Western blot

Tris Base	(25mM) 0.3g
Glycine	(192mM) 14.4g
Methanol	200ml
Distilled water	mix up to 1L

Store at room temperature

Upper Buffer

Tris Base	(0.5M) 6g
Distilled water	mix up to 100ml

Adjust the pH to 6.8 by HCl

Store at 4°C

Wash Buffer for ELISA

Skim milk powder	3g
TBST	100ml

5.2.2 Methods

5.2.2.1 Recombinant protein expression

The competent cells C41 (100 µl) was thaw on ice. One µl of expression construct prepared by my colleague Hilda, the detail information of the construct was shown in Fig. 5.1. (~10-100 ng/µl) was added and mixed gently. A heat shock transformation was preformed. The transformed cells were plated out on LB agar (GibcoBRL) plate with ampicilin (100 ug/ml). After 37°C overnight incubation, 10 different colonies were picked by toothpicks and grew in 3ml culture medium LB with ampicilin (100 ug/ml). The culture was incubated overnight at 37 °C. Next day, the culture medium with antibiotics was aliquoted into different tubes. For each tube the overnight culture in 1:1000 (v/v) was added into the medium, for pilot expression, 3ml medium was used and, for large scale expression, 200ml medium was used. The mixture was incubated at 37°C. When the absorbance reached 0.4-0.6 unit, 0.1M IPTG was added in a ratio of 1:100 (v/v) to induce protein expression. For the control, no IPTG was added. After 8 h incubation at 37 °C, cells were collected by centrifugation (1,000 Xg for 5 min). The pellet was saved and resuspended in 1X TBS. The protein sample was stored at 4 °C until use.

5.2.2.2 Purification of the recombinant protein by Xpress™ System Protein Purification (Invitrogen)

The procedures followed the instruction provided by the supplier. The purification process was under the denaturing conditions. The denaturant used for this system had been changed from 8M urea to 6M guanidine for our own purpose.

5.2.2.3 SDS-PAGE preparation

The following procedure describes the preparation of SDS-PAGE of proteins in a

15% gel. For other gel concentrations, the 40% acrylamide stock and distilled water were adjusted to appropriate ratios. The Bio-Rad Mini-PROTEAN II Cell gel set was used in the experiments. Before casting the gel, the apparatus was washed by 70% EtOH and dried completely. The spacer was inserted in between one larger and one small glass. The glasses with spacers were inserted into the gel stand.

To prepare a 15 % acrylamide of a separating gel, 1.15ml of distilled water, 1.25ml Lower Buffer, 2.5 ml 40% acrylamide stock and 0.25ml 10% sodium dodecyl sulfate (SDS) were mixed in a glass tube. Then 75 µl of 10% ammonium persulfate (APS) and 1.25 µl of N,N,N,N – tetramethyl ethylenediamine (TEMED) was added into the mixture. The mixture was loaded into the gel set immediately in appropriate amount (~4ml). Approximately 0.5ml of isopropanol was loaded on the top of the acrylamide gel to remove the bubble. After the separating gel was formed. The isopropanol was removed by filter paper. Then a 5% of stacking gel was added on the top of the separating gel. The component of the 5% of stacking gel was prepared by mixing 1.65 ml distilled water, 0.75ml Upper Buffer, 0.5ml 40% acrylamide stock, 30 µl of 10% SDS, 40 µl of 10% APS and 3 µl of TEMED. Before the stacking gel formed, the comb was inserted into the gel. After the stacking gel was formed, the comb was removed and the wells were washed with water. The gel was ready to use.

5.2.2.4 SDS-PAGE analysis of proteins

The protein sample was mixed with the 2X Sample Buffer in a ratio of 1:1 (v/v). Then the mixture was denatured in boiling water or at 99 °C in a thermomixer for 15 min. After denaturation, the sample was loaded into the wells of the gel. The gel was run at a constant current of 35 mA until appropriate distance was reached. Then the gel was removed from the gel set and stained in Coomassie Blue Stain solution for 1 h to overnight. The gel was destained in Destaining solution for 1 h or longer. The bands were visible on a

light-box and pictures were taken by scanner (Epson).

5.2.2.5 Western blot analysis

The recombinant protein (refolded or denatured) was analyzed by 15% SDS-PAGE. The SDS-PAGE gel was equilibrated in the transfer buffer for 10-15 min at room temperature. A PVDF membrane (Immobilon pSQ, Minopore) of the same size of the gel was soaked in absolute methanol for few seconds. The membrane and 4 pieces of 3MM Whatmann papers of the same size of the gel were equilibrated in the transfer buffer for 10 min A blotting sandwich was made as follows: (anode side) 2 pieces 3MM Whatmann papers, PVDF membrane, SDS-PAGE gel, and the last 2 pieces 3MM Whatmann papers (cathode side). The sandwich was placed in the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Blotting was preformed under 16 V for 45 min

The blotted membrane was soaked in the blocking buffer and shake for 2 h at room temperature. The rabbit anti-gfPRL polyclonal antibody or rabbit anti-grass crap GH polyclonal antibody was prepared in a 5000-fold dilution in the blocking buffer. The membrane was soaked with the antibody solution and agitated gently on an orbital shaker for overnight at 4°C. The blot was washed with excess amount of TTBS for 15 min twice. Afterward, the blot was incubated with a solution containing goat anti-rabbit IgG secondary antibody (1:1000) conjugated with alkaline phosphatase (Sigma) for 45 min at room temperature. The blot was washed twice with TTBS again and then incubated in a freshly prepared developing solution until color developed. The color development (or the reaction) was stopped by rinsing the blot with large amount of distilled water. Finally, the membrane was air-dried and stored in a plastic bag.

5.2.2.6 Protein refolding

The concentration of the purified recombinant gfPRL was determined by the BCA

Protein Assay Reagent Kit (Pierce). Concentrated and purified recombinant gfPRL was diluted with renaturing solution to lower the guanidine hydrochloride concentration from 6M to 4M and give a final protein concentration of 70 ug/ml. The renaturing solution was added drop by drop, and the mixture was stirred continuously for 48 h at 4°C. Stepwise dialysis was taken place: 24 h in 4M, 2M, 1M, 0.5M, 0M guanidine hydrochloride respectively in 1X TBS, 2mM EDTA, pH8.0.

5.2.2.7 Alkaline Extraction

Hundred pituitary was suspended in 2ml extraction solution containing protease inhibitor cocktail (Roche) and homogenized by Dounce homogenizer with "B" pestle in an ice bath. The homogenate was centrifuged at 1,000 Xg for 5 min at 4 °C. The supernatant was stored in 4 °C and the pellet was resuspended with 1ml extraction solution for the second homogenization as described above. The supernatants were pooled together and stored at –80 °C before use.

5.2.2.8 Size Exclusion Chomatography

Size exclusion chomatography of the pituitary extract was performed by FPLC on a gel filtration column, Superdex 75 (Amersham Pharmacia Biotech)(1.6x60cm, particle size 34μm). The column was first equilibrated with two column volumes of equilibration solution A. Two milliliters of the supernatant was applied to the column which was then eluted with equilibration solution A at a flow rate of 0.2 ml/min The eluted fractions were monitored by absorbance at 280nm.

5.2.2.9 ELISA analysis of the fractions

Fractions from either size exclusion chomatography or anion exchange chomatography was analyzed by ELISA. One hundred microlitres of each fraction was

diluted with 100 ul of carbonate-bicarbonate buffer and incubated in 96 well enzyme immunoassay micro-titre plate (Corning) at 4 °C overnight. The wells was then washed with wash buffer for three times. The plate was incubated with 250 ul blocking buffer at37°C for 2 h. The plate was washed again with wash buffer for three times. Solution containing (1:5000) rabbit anti-gfPRL polyclonal antibody or (1:5000) rabbit anti-gfGH polyclonal antibody was prepared and added to each well with 200 ul. The plate was incubated at 37 °C for 2 h. The plate was washed again with wash buffer for three times and incubated with 200 ul (1:1000) goat anti-rabbit IgG with alkaline phosphatase conjugated secondary antibody (Sigma). The plate was incubated at 37°C for 2 h. The plate was washed with wash buffer for three times. Two hundreds pNPP substrate solution was added to each well and incubated in the dark at 37 °C for yellow color development. The plate was read in Microplate Reader (Bio-Rad) at 405nm.

5.2.2.10 Anion Exchange Chomatography

Pooled fractions containing the native gfPRL or native gfGH was applied to the FPLC on an anion exchange column, Mono Q (Amersham Pharmacia Biotech)(0.5x5 cm, particle size 10 μm) for further purification. The column was first equilibrated with 5 column volumes of equilibration solution B. After loading 0.5 ml the fraction to the column, elution was performed with a linear gradient of elution solution containing 1 M NaCl at a flow rate of 0.5 ml/min The fractions were monitored by absorbance at 280 nm.

5.3 Results

5.3.1 Prokaryotic expression of recombinant gfPRL

The expression construct containing gfPRL coding sequences was successfully transformed into *E. coli* strain, C41(DE3). Ten of clones were randomly selected and induced by 10mM IPTG (8h) for small-scale (3ml) expression. The protein expression level was analyzed by SDS-PAGE (Fig.5.2). Expected size of the recombinant gfPRL is around 25kDa. Lane 3 showed the highest expression level and lane 10 uninduced control. The corresponding clone of lane 3 was selected for large-scale expression (200ml).

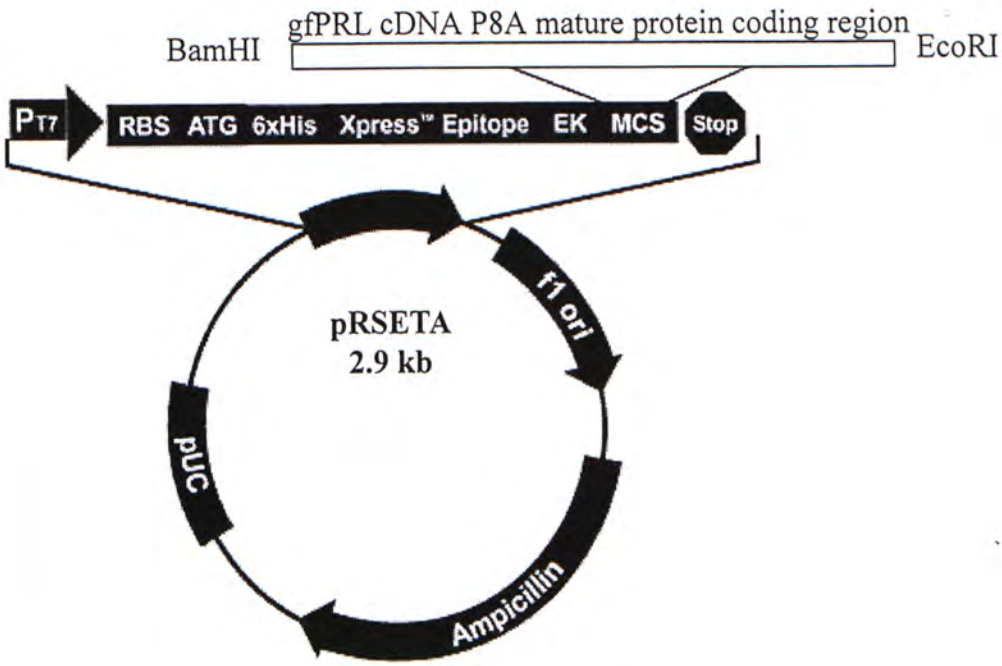


Fig. 5.1. The gfPRL expression construct.

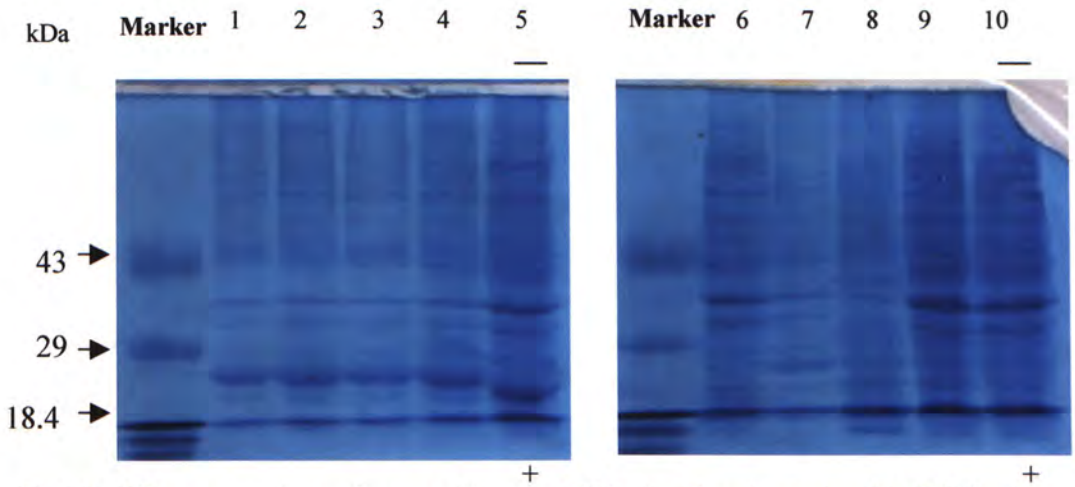


Fig.5.2. Pilot expression of recombinant gfPRL in *E. coli.* strain C41(DE3). Expression vector pRSETA carrying gfPRL coding sequences was transfected into C41(DE3) cells. The transformed cells were induced by 10mM IPTG for 8 h. The cell lyates were collected and boiled in sample buffer for 5 min They were analyzed by SDS-PAGE. Lane 1 is uninduced control and lane 2 to lane 10 came from different transformed colonies. Low molecular weight marker was used.

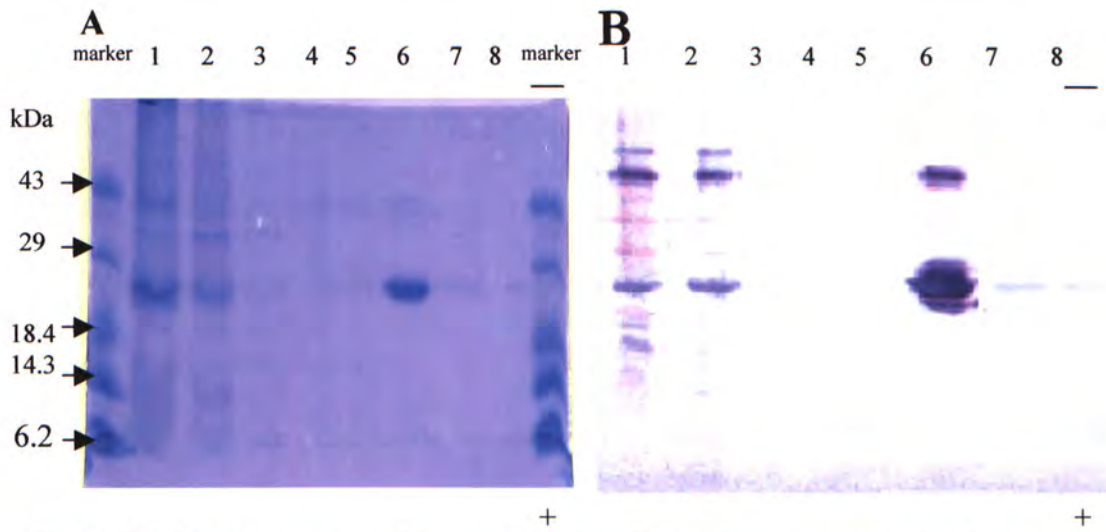


Fig.5.3. Purification of recombinant gfPRL by affinity chomatography. A. The recombinant gfPRL was solubilized by guanidinium lysis buffer and loaded onto the Ni2+ charged resin column. The elution from each purification step were analyzed by SDS-PAGE. B. Western blot analysis of the purified gfPRL. The proteins were transferred to PVDF membrane and blotted by rabbit anti-gfPRL polyclonal antibody (1:5000). Lane 1 is the cell lyate in guanidinium lysis . Lane2 and 3 are the elution after washing by denaturing binding buffer pH7.8; Lane 4 is the elution after washing by denaturing wash buffer pH6.0; Lane 5 is the elution after washing by denaturing wash buffer pH5.3; Lane 6, 7, and 8 are elutions from denaturing elution buffer pH4.

5.3.2 Purification of recombinant gfPRL: SDS-PAGE, western blot and BCA analysis of purified recombinant gfPRL

Cell culture (200ml) expressing recombinant gfPRL (refers to 5.2.2.1) was collected by centrifugation. Cells were resuspened and lysed in guanidine lysis buffer. Cell lysate was sonicated in order to get rid of nucleotide. It was then centrifuged to separate the cell debris and supernatant.

To purify recombinant gfPRL from supernatant, it was loaded onto Ni2+-chelating column. The column was eluted under descending pH buffer. The elutants from each eluting step were analyzed by SDS-PAGE (Fig. 5.3A). Majority of host proteins were eluted at the beginning elution (pH 5.3-ph 7). The recombinant gfPRL contains 6xHistidine residues at the N-terminal, which binds to Ni2+-chelating resins strongly. They dissociated at low pH buffer (pH 4). As a result, a highly purified recombinant gfPRL was collected.

Rabbit anti-goldfish PRL polyclonal antibody binded to the purified gfPRL and gave a strong signal. This confirmed that the recombinant gfPRL was purified (Fig.5.3B). The yield of recombinant gfPRL is 750 μg/ml.

5.3.3 Purification of native gfPRL and gfGH: Native hormone purification by size exclusion chomatography

The pituitary extracts loaded onto the gel filtration column Superdex75 and the elution was monitored by absorbance at 280nm. The elution profile is shown in Fig.5.4 and several peaks were detected. The collected fractions were analyzed by ELISA using rabbit anti-gfPRL polyclonal antibodies and rabbit anti-grass carp GH polyclonal antibodies respectively and the result is shown in Fig. 5.5. Two major peaks of gfGH and gfPRL profiles located within 32-36 fractions. The fraction 34 and 35 were pooled together for native gfPRL purification while fraction 31, 32 and 33 were pooled together for native gfGH

purification.

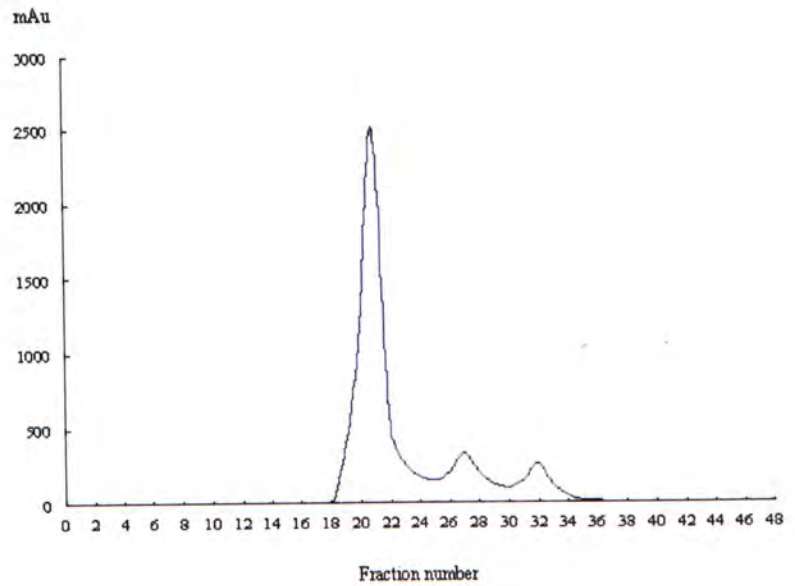


Fig.5.4. Size exclusion chomatography of the goldfish pituitary extract on Superdex 75 column. The goldfish pituitary extract was fractionated by size exclusion chomatography on a Superdex 75 column, column size: 1.6x60 cm; particle size:34 mm. 50mM ammonium bicarbonate (pH8.0) buffer was used for equilibration and elution. The flow rate was maintained at 0.2 ml/min

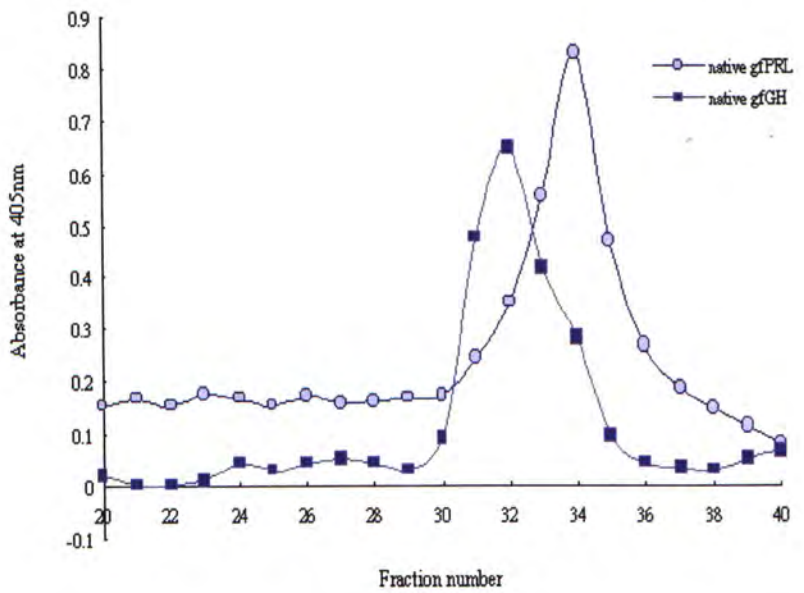


Fig.5.5. The amount of native PRL and GH in different fractions eluted from Superdex 75 column as detected by ELISA. Two ELISA analysis were carried out using rabbit anti-gfPRL polyclonal antibody (1:5000) and rabbit anti-grass crap GH polyclonal antibody (1:5000) respectively. Mouse anti-rabbit IgG conjugated with alkaline phosphatase was used as secondary antibody. The absorbance was measured at 405nm.

5.3.4 Native gfPRL purification by anion exchange chomatography

The pooled fraction was load onto anion exchange column Mono Q and monitored by reading absorbance at 280nm. Fig. 5.6. showed the elution profile of native gfPRL purification. Two discrete major peaks were detected in fraction 10 and 16. Each fraction was analyzed by ELISA using rabbit anti-gfPRL polyclonal antibody. Fraction 16 showed the highest absorbance reading (Fig. 5.6B)

Fig. 5.7A showed the elution profile of native gfGH purification. A single major peak was detected in fraction 15. Each fraction was analyzed by ELISA using rabbit anti-grass carp GH polyclonal antibody. Fraction 15 showed the highest absorbance reading (Fig. 5.7B)

SDS-PAGE, BCA and western blot analysis of purified gfPRL and gfGH. The purified hormones were analyzed by SDS-PAGE and western blot (Fig. 5.8). Rabbit anti-gfPRL polyclonal antibody and rabbit anti-grass carp GH polyclonal antibody were used to detect the native gfPRL and gfGH respectively. The size of the native gfPRL and gfGH are around 21 and 22 kDa respectively. The protein concentration of native gfPRL and gfGH were determined by BCA and they are 70 μg/ml and 100 μg/ml respectively.

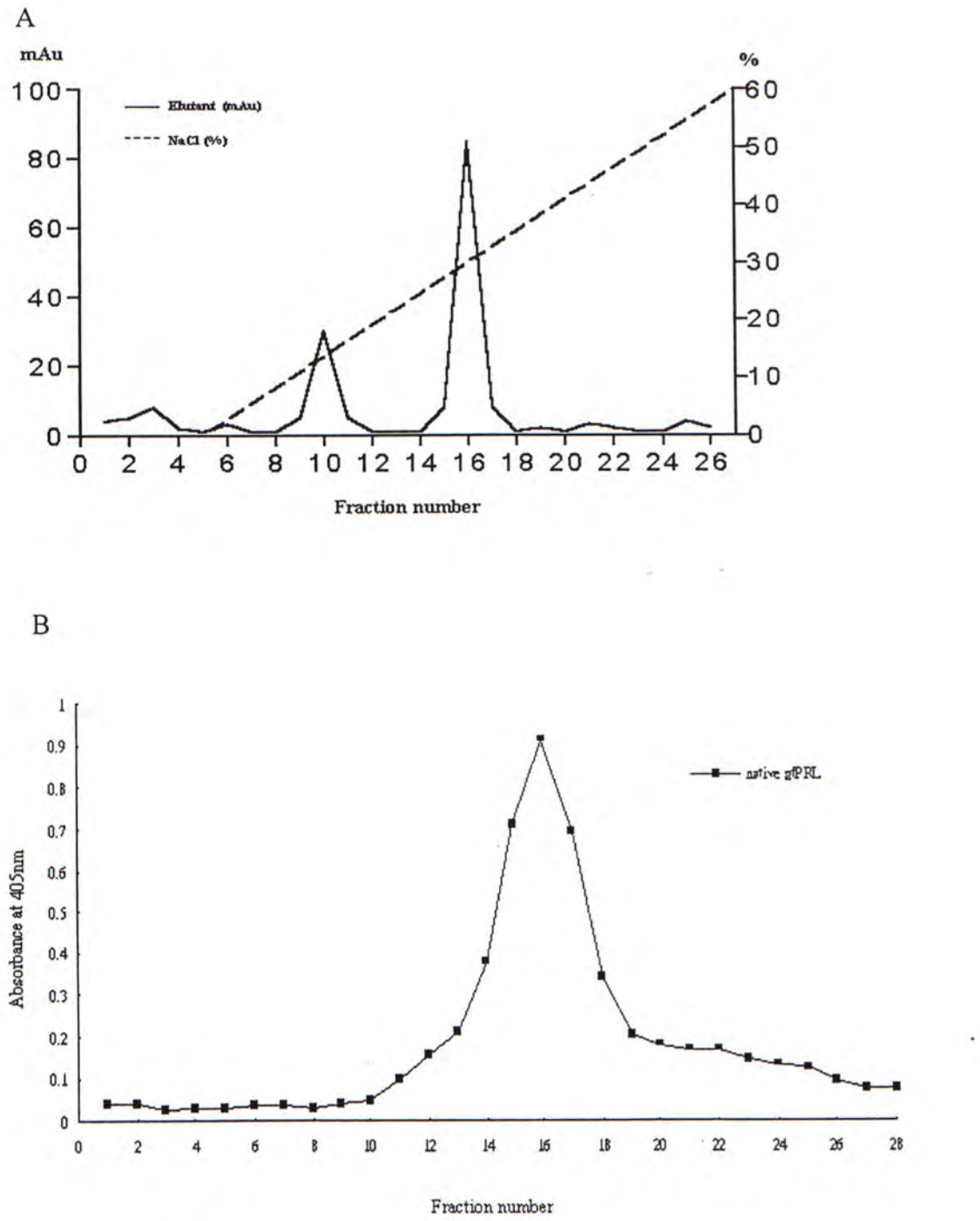
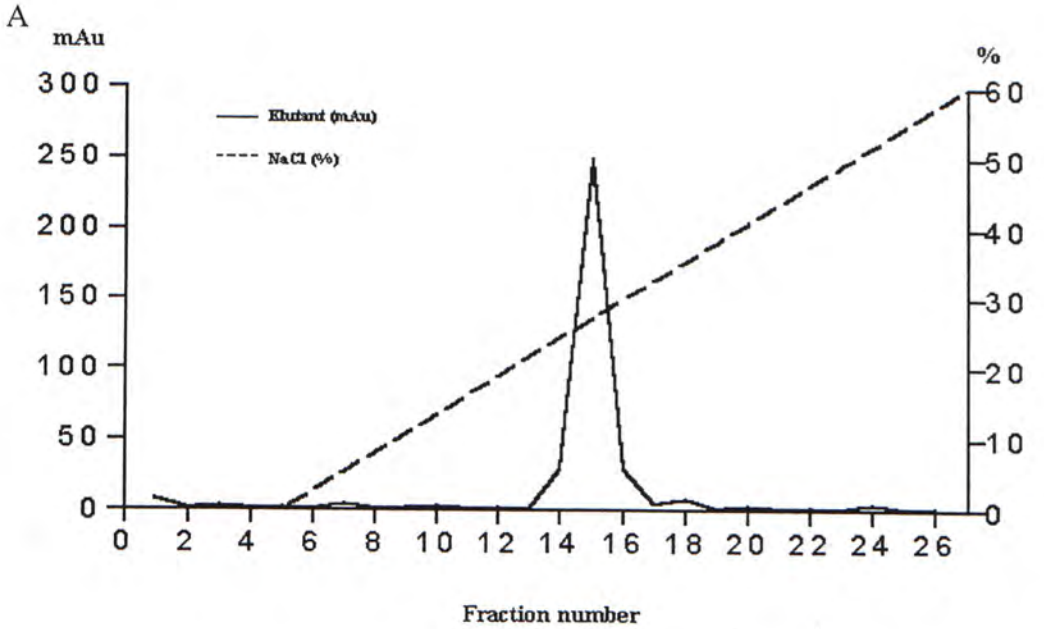


Fig.5.6 A. Purification of native gfPRL. Anion exchange chomatography of pooled fraction 34 and 35 from Superdex 75 column on a Mono Q column. The protein was eluted with linear gradient of 1M NaCl in 50mM ammonium bicarbonate, pH7.6, at a flow rate of 0.5ml/min. B. Analysis of the amount of native gfPRL in different fractions eluted from Mono Q column. ELISA was carried out using rabbit anti-gfPRL polyclonal antibody (1:5000). Mouse anti-rabbit IgG conjugated with alkaline phosphatase was used as secondary antibody. The absorbance was measured at 405nm.

A



B

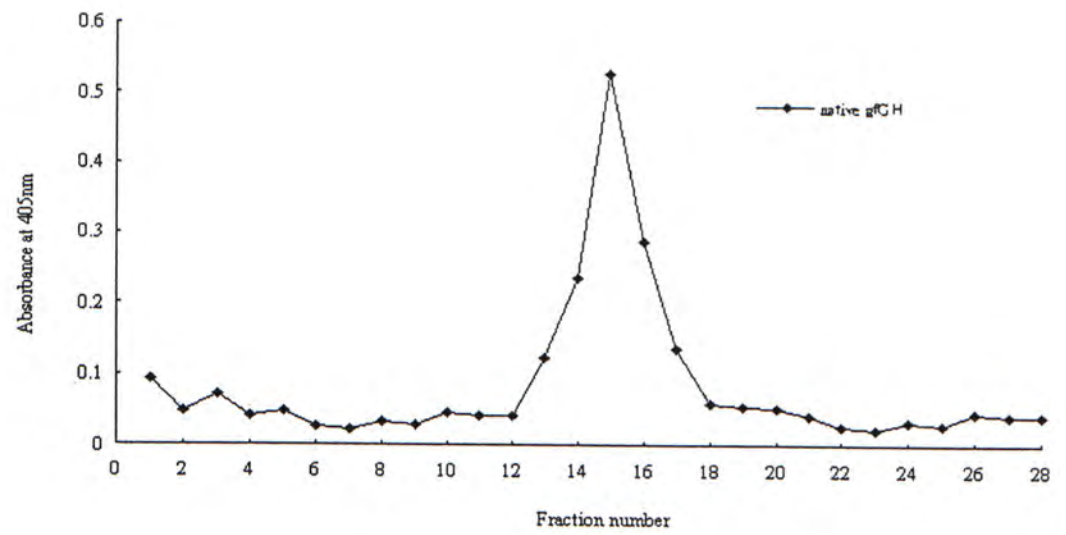


Fig.5.7A. Purification of native gfGH. Anion exchange chomatography of pooled fraction 31, 32 and 33 from Superdex 75 column on a Mono Q column. The protein was eluted with linear gradient of 1M NaCl in 50mM ammonium bicarbonate, pH7.6, at a flow rate of 0.5ml/min. B. Analysis of the amount of native gfGH in different fractions eluted from Mono Q column. ELISA was carried out using rabbit anti-gfGH polyclonal antibody (1:5000). Mouse anti-rabbit IgG conjugated with alkaline phosphatase was used as secondary antibody. The absorbance was measured at 405nm.

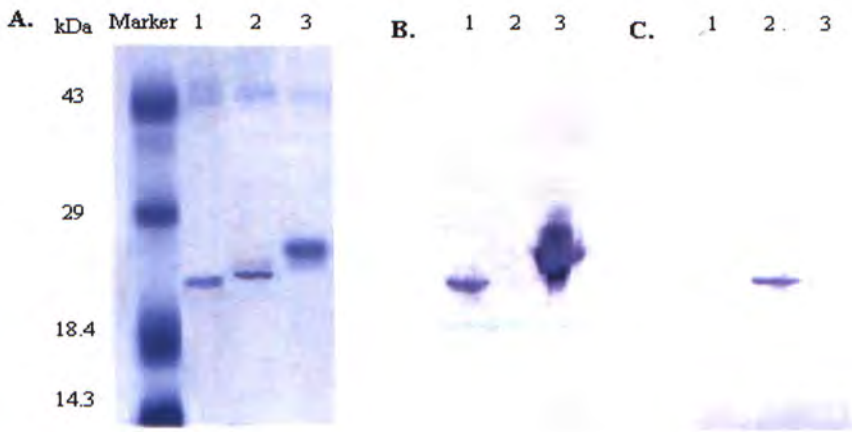


Fig.5.8. SDS-PAGE analysis of purified native gfGH and gfPRL. A. Purified gfGH and gfPRL was analyzed by SDS-PAGE under reducing condition. Lane 1 is the native gfPRL; Lane 2 is the native gfGH; Lane 3 is the recombinant gfPRL. B. Western blot analysis of purified native gfGH and gfPRL. Proteins were transferred to PVDF membrane and blotted by rabbit anti-gfPRL polyclonal antibody (1:5000). C. Proteins were blotted by rabbit anti-grass carp GH polyclonal antibody (1:5000).

5.3.5 Study the biological activity of refolded recombinant gfPRL

The refolded recombinant gfPRL was used to stimulate the β-casein promoter fused with luciferase reporter gene in CHO cells expressing gfPRL receptor. Recombinant gfPRL stimulates luciferase activity by 1.5 and 2 fold at concentration 0.625 μg/ml and 1.25 μg/ml respectively (Fig. 5.9).

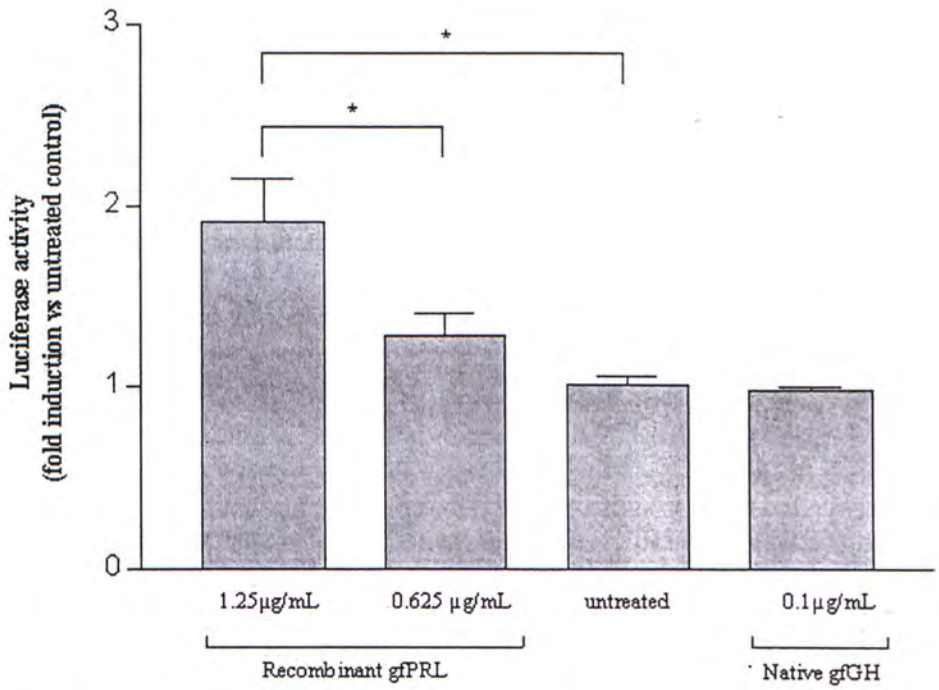


Fig.5.9. Induction of luciferase activities by refolded recombinant gfPRL. b-casein promoter fused with luciferase reporter gene and co-transfected (500 ng) with mSTAT5a expression vector (300 ng) into CHO cells stably expressing gfPRL receptor. The transfected cells were incubated with 1.25 mg/ml and 0.625 mg/ml recombinant gfPRL and 0.1 mg/ml native gfGH. The untreated and GH treated groups act as negative controls. The luciferase activities of each treated group were compared with untreated group. N=4 Vertical bars=S.E.M . * student's t-test $P<0.05$

Discussion

5.4.1 Prokaryotic expression of recombinant gfPRL

Escherichia coli is one of the most successful vehicles for over-expression of both prokaryotic and eukaryotic proteins (Iost and Dreyfus, 1995; Hockney, 1994) and has been widely used in both industry and laboratory (Swartz, 2001). It provides an inexpensive, convenient and efficient recombinant protein production procedure.

In the *E. coli* BL21(DE3) host strain, the T7 polymerase is produced from the λ-lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG-inducible lac UV5 promoter while the target gene is positioned downstream of the bacteriophage T7 late promoter of a expression vector. This system has been employed successfully for over-production of many globular proteins, but in many other cases significant over-production cannot be achieved because of the toxicity of over-expression (George *et al.*, 1994; Studier *et al.*, 1990). A mutant host C41(DE3) derived from BL21(DE3) which grew to high saturation cell density and produced the protein as inclusion bodies at an elevated level without toxic effect (Miroux and Walker, 1996). C41(DE3) has a relatively low transcription activity compared to BL21(DE3), which helps to prevent coupling of transcription and translation (toxic effect). In this experiment, C41(DE3) was used in order to obtain a high yield of recombinant gfPRL.

The expression vector pRSETA (Invitrogen) is pUC-derived expression vectors which replicates few hundred copies per cell and is suitable for high level protein expression. The transcription of the vector is regulated by T7 promoter. The DNA insert is positioned downstream and in frame with a sequence that encode six histidine residues which is a metal binding domain for single step purification of recombinant protein by affinity chomatography.

A high level of expression of recombinant gfPRL was obtained after eight hours with IPTG induction. They formed inclusion bodies due to over-expression in heterologous system. When eukaryotic protein expresses in prokaryotic system, improper protein folding happens. Folding intermediates accumulate and co-precipitate with other impurities such as host proteins and nucleotides to form inclusion body (Hartley and Kane, 1988). In general, inclusion bodies are held together by non-covalent forces, mostly hydrophobic interactions (Bowden *et al.*, 1991). The cytoplasm of *E. coli.* contains a rather high concentration of glutathione which normally prohibits the formation of disulfide bonds (Stewart *et al.*, 1990). As a result, proteins requiring disulfide bond formation for folding usually aggregate when they are expressed in the cytoplasm of *E. coli.*. However, there are many advantages to expressing recombinant proteins in inclusion body form from a downstream processing prospective. The protein of interest usually represents more than 50% of the total protein contained in inclusion bodies. Purification sequences for inclusion body proteins generally require fewer steps than sequences for comparable proteins expressed in soluble form, which tends to reduce time and reduce losses.

5.4.2 Purification of recombinant gfPRL

To recover the biological activity of the recombinant gfPRL, isolation and purification from inclusion bodies as well as refolding are the necessary processes. Guanidine hydrochloride is the denaturant to disrupt the inter-molecular interactions in inclusion bodies. It is preferable to urea as the presence of isocyanate in urea solutions can cause irreversible modifications of amino or thiol groups of the polypeptides (Gerding *et al.*, 1971; Hagel *et al.*, 1971). The solublized recombinant gfPRL possessing 6x histine residues at N-terminal that has a high affinity ($Ka=10^{13}$) toward Ni^{2+}-chelating resin at pH7.8 in the presence of guanidine hydrochloride (Hochuli, 1988). This characteristic favors the isolation of

recombinant gfPRL from other contaminants by Ni^{2+}-chelating resin column. SDS-PAGE and western blot analysis showed that the purified recombinant gfPRL are of 25 kDa and 50 kDa respectively. They are larger than the expected size of gfPRL (21 kDa) because of extra histidine addition and dimerization.

The extra histidine in the recombinant gfPRL was not removed. It is because reports showed that histidine tag is non-immunogenic and uncharged at physiological pH and will not affect the secretion. Besides, compartmentalization or folding of the protein will not be altered (Janknecht *et al.*, 1991). As a result, purified recombinant gfPRL undergo refolding directly.

5.4.3 Refolding of recombinant gfPRL

Refolding is a process involved in the removal of denaturant and formation of intra-molecular disulfide bridge. In our study, it is done by dilution, dialysis and diafiltration (Levine *et al.*, 1995; Werner *et al.*, 1994). The purified recombinant gfPRL was diluted to 70 ug/ml to avoid aggregation of partially folded polypeptides during dialysis. A steadily and gradually decreased guanidine hydrochloride gradient between the environment and the recombinant gfPRL solution was setup as described in the material and method section. In addition, dialysis was performed at 4 °C to provide a longer time and slower folding rate for polypeptide to achieve a more kinetic stable state. Thiol reagent β-mercaptoethanol and a chelating agent EDTA were also used as reducing agents in the cleavage of disulfide bonds. A steadily oxidative potential was maintained by addition of both reduced (GSH) and oxidized glutathione (GSSG) to accelerate the rearrangement of disulfide bonds by facilitating thiol-disulfide exchange (Wetlaufer, 1984). Non-detergent sulphobetaine (NDSB-256) was used to prevent aggregation and promote efficient refolding of the recombinant gfPRL (Goldberg *et al.*, 1996). NDSB has two opposite charges separated by a

carbon bridge and thus forms dipole. It weakens hydrophilic and hydrophobic protein-protein interactions, to minimize protein aggregation and improper folding. Moreover, NDSB-protein interaction competes with internal polypeptides bonding formation, which facilitates proper folding of protein itself.

5.4.4 Purification of native gfPRL

Up to present, PRLs purified have molecular weight of 21-23 kDa (Suzuki *et al.*, 1991; Miyajima *et al.*, 1988; Prunet and Houdebine, 1984; Kawauchi *et al.*, 1983). Our result is consistent with these findings.

5.4.5 Study the biological activity of recombinant gfPRL

PRL is a mitogen for a number of cell types such as lymphoid and myeloid, through binding to its receptor and induction of tyrosine phophorylation and activation of the cytoplasmic tyrosine kinase JAK2, followed by recruitment of signal transducers and activators of transcription (STATs)(Bole-Feysot *et al.*, 1998). PRL has been shown to promoter differentiation of CHO cells transfected with the rabbit PRLR (Bignon *et al.*, 1995). CHO cells stably expressing gfPRL receptor (Tse *et al.*, 2000) was used to demonstrate the functional interaction between recombinant gfPRL and gfPRLR. . Construct of β-casein gene promoter fused with luciferase reporter gene and mouse STAT5a expressing vector were co-transfected into CHO cells expressing gfPRL receptor. β-casein gene transcription is controlled primarily by a composite response element (CoRE) that integrates signaling from the lactogenic hormones, PRL, insulin, and hydrocortisone, in mammary epithelial cells (Rosen *et al.*, 1999). This CoRE contains binding sites for STAT5 (signal transducer and activator of transcription 5) and C/EBPbeta (CCAAT/enhancer-binding protein-beta) and several half-sites for glucocorticoid receptor (GR).

The refolded recombinant gfPRL binds to the gfPRL receptor and activate of

STAT5a protein (Gouilleux *et al.*, 1995). STAT5a binds to β-casein promoter and stimulates transcription activity of luciferase gene. Our result demonstrated that the refolded recombinant gfPRL induced luciferase activity and is biologically active. The fold-induction by recombinant gfPRL can be further increased by increasing the time of incubation as well as the amount of mSTAT5a vector transfection.

5.5 Conclustion

Recombinant gfPRL was successfully expressed in E. coli.. After purification and refolding process, the recombinant gfPRL has regained its biological activity as demonstrated in luciferase assay in CHO-K1 cell expressing gfPRLR. In addition, the native gfPRL and gfGH have been purified and analysed in both western blot and ELISA.

All in all, our investigation on the functions of prolactin other than osmoregulation in fresh water teleost is still far from the conclusion. However, the results generated from this project may provide some insight for the future investigation.

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